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(54) Title: GENE ENCODING MNR2 AND USES THEREOF

(57) Abstract

This invention provides isolated nucleic acids encoding a motor neuron restricted MNR2 protein, and a homeobox HB9 protein. Also provided are purified MNR2 and HB9 proteins, antibodies recognizing these proteins, transgenic animals expressing these proteins, and functionally equivalent analogs of these proteins. Finally, methods are disclosed for inducing differentiation of somatic motor neurons, and for treating diseases related to a lack of normally functioning motor neurons, neurodegenerative diseases, acute nervous system injury, and neuromuscular disease.

GENE ENCODING MNR2 AND USES THEREOF

5   This application claims priority of and is a continuation-
       in-part of U.S. Serial No. 09/162,524, filed September 29,
       1998, the content of which is hereby incorporated by
       reference.

10   The invention disclosed herein was made with support under
       National Institute of Health training grant No. 5T32GM07367,
       U.S. Department of Health and Human Services. Accordingly,
       the United States Government has certain rights in this
       invention.

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       Throughout this application various publications are
       referred to within parenthesis. Full citations for these
       publications may be found at the end of the specification
       immediately preceding the claims. The disclosures of these
20   publications, in their entireties, are hereby incorporated
       by reference into this application in order to more fully
       describe the state of the art to which this invention
       pertains.

25   **Background of the Invention**

       Sonic hedgehog signaling controls the differentiation of
       motor neurons in the ventral neural tube but the intervening
       steps are poorly understood. A differential screen of a
30   cDNA library derived from a single Shh-induced motor neuron
       has identified a novel homeobox gene, MNR2, expressed by
       motor neuron progenitors and transiently by post-mitotic
       motor neurons. The ectopic expression of MNR2 in neural
       cells initiates a program of somatic motor neuron
35   differentiation characterized by the expression of
       homeodomain proteins, by neurotransmitter phenotype and by
       axonal trajectory. Our results suggest that the Shh-
       mediated induction of a single transcription factor, MNR2,
       is sufficient to direct somatic motor neuron

differentiation.

The assembly of neural circuits in the vertebrate central nervous system (CNS) is initiated by the generation of distinct classes of neurons at characteristic positions. The specification of neuronal identity in the CNS appears to be controlled by inductive signals secreted by embryonic organizing centers (Lumsden and Krumlauf, 1996; Tanabe and Jessell, 1996). These signals appear to define neuronal fates by regulating the expression of cell-intrinsic determinants, many of which are transcription factors (Bang and Goulding, 1996). However, the pathways by which specific inductive signals determine the fate of individual neuronal cell types in the CNS are poorly defined. As a consequence, it is unclear whether there are individual transcription factors assigned, in a dedicated manner, to the specification of particular neuronal subtypes or whether the parallel actions of several factors are required.

Spinal motor neurons constitute one subclass of CNS neuron for which some early differentiation steps have been defined (Pfaff and Kintner, 1998). The differentiation of motor neurons depends on spatial signals provided by Sonic Hedgehog (Shh) secreted from the notochord and floor plate (Marti et al. 1995; Roelink et al., 1995; Tanabe et al., 1995; Chiang et al. 1996; Ericson et al. 1996). Shh acts initially to convert medial neural plate cells into a population of ventral progenitors (Ericson et al., 1996) and later directs the differentiation of ventral progenitors into motor neurons and interneurons at distinct concentration thresholds (Roelink et al. 1995; Ericson et al., 1997). The Shh-induced pathway of motor neuron differentiation appears, however, to operate within the context of an independent program of neurogenesis. Neural progenitors that have been exposed to Shh undergo two or more cell divisions before leaving the cell cycle and acquiring motor neuron properties (Ericson et al., 1996). Over this period, ventral progenitors require continued Shh

signaling, achieving Shh-independence and committing to a motor neuron fate only late in their final division cycle (Ericson et al., 1996).

Cells in the ventral neural tube respond to graded Shh signaling with the establishment of distinct ventral progenitor populations defined by the expression of the homeodomain proteins Pax6 and Nkx2.2 (Ericson et al., 1997). These two progenitor populations generate distinct classes of motor neurons. Pax6^{+} progenitors give rise to somatic motor neurons whereas Nkx2.2[+] progenitors generate visceral motor neurons (Ericson et al. 1997). As these two progenitor populations leave the cell cycle they express different homeodomain proteins that characterize distinct motor neuron subtypes (Tsuchida et al., 1994; Varela-Echavarria et al., 1996; Ericson et al., 1997; Pattyn et al. 1997). The activity of Pax6 is necessary for the differentiation of somatic motor neurons within the hindbrain (Ericson et al., 1997; Osumi et al., 1997) but it appears that its function is indirect, being required to repress the expression of Nkx2.2 (Ericson et al., 1997).

The dispensibility of Pax6 for somatic motor neuron generation implies the existence of additional genes that determine somatic motor neuron identity. Moreover, the late commitment of progenitors to a somatic motor neuron fate suggests that the onset of expression of such genes occurs only during the final division cycle of motor neuron progenitors. To identify such determinants a screen for genes expressed by somatic motor neuron progenitors was performed and described, here is the characterization of a novel homeobox gene, MNR2.

MNR2 is expressed selectively by Pax6^{+} motor neuron progenitors and persists transiently in post-mitotic somatic motor neurons. The ectopic expression of MNR2 in vivo is sufficient to activate a program of somatic motor neuron differentiation characterized by the expression of several

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homeodomain proteins and <u>Choline Acetyltransferase (ChAT)</u>, by the autoactivation of <u>MNR2</u> and by the extension of axons into ventral roots. This program of motor neuron differentiation is accompanied by the repression of spinal interneuron fates. Thus, the Shh-triggered differentiation of ventral progenitor cells into somatic motor neurons may be directed by the expression of a single homeodomain protein, MNR2.

Introduction

The ability of neurons to form selective neuronal circuits is a function of the molecular properties that they acquire at early stages of their differentiation. The molecular features that distinguish individual classes of neurons appear to control the pattern of axonal projections, the formation of target connections and the expression of specific chemical transmitters. The emergence of a coherent neuronal phenotype is a protracted process and is thought to involve progressive restrictions in the developmental potential of both neural progenitor cells and post-mitotic neurons (Cepko 1999; Edlund and Jessell, 1999). In the peripheral nervous system, convergent programs of transcription factor expression have been suggested to coordinate pan-neuronal properties with more specific aspects of neuronal subtype identity, notably neurotransmitter synthesis and trophic factor sensitivity (Lo et al., 1998, 1999; Pattyn et al., 1999; Goridis and Brunet, 1999).

When and how neuronal subclasses in the central nervous system acquire their specialized functional properties is less well understood. Studies of the differentiation of spinal motor neurons (MNs) have provided some insight into the steps that confer neuronal subtype identity within the central nervous system. Physiological and anatomical studies have revealed that spinal MNs exhibit several levels of organization and function (Landmesser, 1978 a, b) and these have a molecular correlate in the selective patterns of

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expression of different families of transcription factors
(Tanabe and Jessell, 1996; Goulding, 1998). Members of the
LIM homeodomain (LIM-HD) protein family define aspects of
the generic and columnar identities of spinal MNs (Ericson
5   et al., 1992; 1996; Tsuchida et al., 1994; Sharma et al.,
1998). In addition, many of the MN pools that innervate
individual muscles in the limb can be defined by the
expression of ETS domain proteins (Lin et al., 1998). The
analysis of neuronal fate changes that result from the
10   misexpression or inactivation of certain of these nuclear
factors has lent support to the idea that they have critical
roles in the specification of MN identity (Tanabe et al.,
1998: Sharma et al., 1998; see Appel, 1999).

15   Some of the earlier events that specify the differentiation
of neural progenitors into MNs have also been defined. The
differentiation of MNs is initiated when progenitor cells
located in the ventral half of the neural tube acquire
distinct identities in response to the graded signaling
20   activity of Sonic hedgehog (Shh) (Ericson et al., 1996,
1997a,b; Briscoe et al., 1999). The final division of MN
progenitors in chick is marked by the onset of expression of
two homeodomain proteins, MNR2 and Lim3 (Lhx3) which appear
to have distinct roles in MN differentiation (Ericson et
25   al., 1997a; Tanabe et al., 1998; Sharma et al., 1998). MNR2
expression is restricted to MN progenitors whereas Lim3 is
expressed by progenitor cells that give rise to an adjacent
population of V2 interneurons (Ericson et al., 1997a; Tanabe
et al., 1998). In chick, the ectopic expression of MNR2 is
30   sufficient to direct the differentiation of neural cells
into MNs and to suppress V2 interneuron generation (Tanabe
et al., 1998). In contrast, ectopic expression of Lim3
alone appears to promote the generation of V2 interneurons
(Tanabe et al., 1998). These results suggest that MNR2 has
35   a role in specifying whether ventral progenitors that
express Lim3 generate MNs rather than V2 neurons.

The function of many of the other transcription factors

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whose expression is restricted to MNs has not yet been addressed. Amongst these, the homeobox gene Hb9 (Harrison et al., 1994; Ross et al., 1998) is a selective marker of MNs in the developing spinal cord (Pfaff et al., 1996; Saha et al., 1997; Tanabe et al., 1998). Strikingly, HB9 possesses a homeodomain virtually identical to that of MNR2. Moreover, the ectopic expression of HB9 in chick has been shown to mimic the MN-inducing and V2 interneuron repressive activities of MNR2 (Tanabe et al., 1998; unpublished data). In contrast to MNR2, however, the expression of HB9 in chick is excluded from ventral progenitor cells and is restricted to post-mitotic MNs (Tanabe et al., 1998), suggesting that it has a later role in the differentiation of post-mitotic MNs. Further insight into the developmental roles of MNR2 and HB9, however, requires an analysis of MN differentiation in embryos that lack the function of these homeodomain proteins.

To begin to address this issue we have examined MN development in mice in which the Hb9 gene has been inactivated by targeted mutation. In mice lacking Hb9 function, MNs are generated on schedule and in normal numbers. However, soon after MNs have left the cell cycle, there is a dramatic change in the program of MN differentiation. Most strikingly, MNs transiently express transcription factors normally characteristic of V2 interneurons. In addition, and perhaps as a consequence, the transcription factor codes that define the columnar and pool identities of spinal MNs are markedly disrupted. These defects in the transcription factor profile of MNs are accompanied by abnormal MN migratory patterns, by errors in motor axon projections and by defects in the innervation of certain target muscles. Together, these results provide evidence that HB9 has a critical role in the consolidation of MN identity, in particular in repressing the expression of V2 interneuron character.

The initial stages of pancreatic development occur early

during mammalian embryogenesis (Wessells et al. 1981) but the genes governing this process remain largely unknown. The homeodomain protein IPF1/PDX1 is expressed in the developing pancreatic anlagen from the -10 somite stage (Ohlsson et al.

5   1993; Ahlgren et al. 1996) and mutations in the IPF1/PDX1 gene prevent the development of the pancreas (Ahlgren et al. 1996; Jonsson et al. 1994; Offield et al. 1996; Harrison et al. 1994). However, the initial stages of pancreatic development still occur in Ipf1/Pdx1 deficient mice (Ahlgren

10   et al. 1993). Hb96 is a homeobox gene that in humans has been linked to dominant inherited sacral agenesis (Ross et al. 1998) and we show here that HB9 is expressed at early stages of mouse pancreatic development and later in differentiated -cells. Hb9 has an essential function in the

15   initial stages of pancreatic development. In absence of Hb9 expression, the dorsal region of the gut epithelium fails to initiate a pancreatic differentiation program. In contrast, the ventral pancreatic endoderm develops but exhibits a later and more subtle perturbation in -cell differentiation

20   and in islet cell organisation. Thus, dorsally Hb9 is required for specifying the gut epithelium to a pancreatic fate and ventrally for ensuring proper -cell differentiation.

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Summary of the Invention

This invention provides an isolated nucleic molecule encoding a motor neuron restricted pattern, MNR2, protein.

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This invention provides a vector which comprises the isolated nucleic acid encoding a motor neuron restricted pattern, MNR2, protein.

10   This invention provides a host cell containing the vector which comprises the isolated nucleic acid encoding a motor neuron restricted pattern, MNR2, protein.

This invention provides a method of producing a polypeptide
15   having the biological activity of a mammalian MNR2 which comprises growing host cells selected from a group consisting of bacterial, plant, insect or mammalian cell, under suitable conditions permitting production of the polypeptide.

20

This invention provides an isolated nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the nucleic acid molecule encoding a motor
25   neuron restricted pattern, MNR2, protein.

This invention provides an antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to an mRNA molecule encoding a MNR2 protein.

30

This invention provides a monoclonal antibody directed to an epitope of an MNR2 protein.

This invention provides a purified MNR2 protein.

35

This invention provides a method of inducing differentiation somatic motor neurons which comprises expressing MNR2 protein in neural progenitor cells.

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This invention provides a transgenic nonhuman mammal which comprises an isolated nucleic acid encoding a motor neuron restricted pattern, MNR2, protein, is a DNA molecule.

5   This invention provides a method of determining physiological effects of expressing varying levels of MNR2 protein in a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman animals, each a transgenic nonhuman mammal, each nonhuman mammal expressing

10   a different amount of MNR2 protein.

This invention provides a method of producing an isolated purified MNR2 protein which comprises: a) inserting a nucleic acid molecule encoding a MNR2 protein into a

15   suitable vector; b) introducing the resulting vector into a suitable host cell; c) selecting the introduced host cell for the expression of the MNR2 protein; d) culturing the selected cell to produce the MNR2 protein; and e) recovering the MNR2 protein produced.

20
This invention provides a method of inducing differentiation of somatic motor neurons in a subject comprising administering to the subject the purified MNR2 protein in an amount effective to induce differentiation of somatic motor

25   neurons in the subject.

This invention provides a pharmaceutical composition comprising a purified MNR2 protein and a pharmaceutically acceptable carrier.

30
This invention provides a method for treating a subject afflicted with an abnormality associated with a lack of one or more normally functioning motor neurons which comprises introducing an amount of a pharmaceutical composition

35   comprising a purified MNR2 protein and a pharmaceutically acceptable carrier effective to generate somatic motor neurons from undifferentiated motor neuron precursor cells in the subject, thereby treating the subject afflicted with

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the abnormality associated with the lack of one or more normally functioning motor neurons.

This invention provides a method of treating a subject afflicted with a neurodegenerative disease which comprises introducing an amount of a pharmaceutical composition which comprises a purified MNR2 protein and a pharmaceutically acceptable carrier effective to generate somatic motor neurons from undifferentiated precursor motor neuron cells in the subject, thereby treating the subject afflicted with the neurodegenerative disease.

This invention provides for a method of treating a subject afflicted with an acute nervous system injury which comprises introducing an amount of a pharmaceutical composition which comprises a purified MNR2 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated precursor motor neuron cells in a subject, thereby treating the subject afflicted with the acute nervous system injury.

This invention provides for a method of treating a subject afflicted with an acute nervous system injury, wherein the acute nervous system injury is localized to a specific central axon which comprises surgical implantation of a pharmaceutical composition comprising a MNR2 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated precursor motor neuron cells located proximal to the specific central axon, so as to alleviate the acute nervous system injury localized to a specific central axon, thereby treating the subject afflicted with the acute nervous system injury.

This invention provides a method for diagnosing a chronic neurodegenerative disease associated with the expression of a MNR2 protein in a sample from a subject which comprises: (a) obtaining DNA from the sample of the subject suffering from the chronic neurodegenerative disease; (b) performing

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a restriction digest of the DNA with a panel of restriction enzymes; (c) separating the resulting DNA fragments by size fractionation; (d) contacting the resulting DNA fragments with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a MNR2 protein, wherein the sequence of a nucleic acid molecule encoding a MNR2 protein is linked at a specific break point to a specified nucleic acid sequence and labeled with a detectable marker; (e) detecting labeled bands which have hybridized to the nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a MNR2 protein, wherein the sequence of a nucleic acid molecule encoding a MNR2 protein is linked at a specific break point to a specified nucleic acid sequence to create a unique band pattern specific to the DNA of subjects suffering from the chronic neurodegenerative disease; (f) preparing DNA obtained from a sample of a subject for diagnosis by steps (a-e); and (g) comparing the detected band pattern specific to the DNA obtained from a sample of subjects suffering from the chronic neurodegenerative disease from step (e) and the DNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the chronic neurodegenerative disease if the patterns are the same.

This invention provides a method for diagnosing a chronic neurodegenerative disease associated with the expression of a MNR2 protein in a sample from a subject which comprises: (a) obtaining RNA from the sample of the subject suffering from chronic neurodegenerative disease; (b) separating the RNA sample by size fractionation; (c) contacting the resulting RNA species with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a MNR2 protein, wherein the sequence of a nucleic acid

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molecule encoding a MNR2 protein is linked at a specific break point to a specified nucleic acid sequence and labeled with a detectable marker; (d) detecting labeled bands which have hybridized to the RNA species to create a unique band pattern specific to the RNA of subjects suffering from the chronic neurodegenerative disease; (e) preparing RNA obtained from a sample of a subject for diagnosis by steps (a-d); and (f) comparing the detected band pattern specific to the RNA obtained from a sample of subjects suffering from the chronic neurodegenerative disease from step (d) and the RNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the chronic neurodegenerative disease if the patterns are the same.

This invention provides a functionally equivalent analog of MNR2 that induces MNR2 differentiation of neural progenitor cells.

This invention provides a functionally equivalent analog of MNR2 that prevents MNR2 differentiation of neural progenitor cells.

This invention provides a method of treating a subject afflicted with a neuromuscular disease which comprises introducing an amount of a pharmaceutical composition comprising a purified MNR2 protein and a pharmaceutically acceptable carrier effective to activate acetylcholine to activate muscle cells.

This invention provides an isolated nucleic molecule encoding a homeobox, HB9, protein.

This invention provides a vector which comprises the isolated nucleic acid encoding a homeobox, HB9, protein.

This invention provides a host cell containing the vector

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which comprises the isolated nucleic acid encoding a homeobox, HB9, protein.

This invention provides a method of producing a polypeptide having the biological activity of a mammalian HB9 which comprises growing host cells selected from a group consisting of bacterial, plant, insect or mammalian cell, under suitable conditions permitting production of the polypeptide.

This invention provides an isolated nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the nucleic acid molecule encoding a homeobox, HB9, protein.

This invention provides an antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to an mRNA molecule encoding a HB9 protein.

This invention provides a monoclonal antibody directed to an epitope of an HB9 protein.

This invention provides a purified HB9 protein.

This invention provides a method of inducing differentiation somatic motor neurons which comprises expressing HB9 protein in neural progenitor cells.

This invention provides a transgenic nonhuman mammal which comprises an isolated nucleic acid encoding a homeobox, HB9, protein, is a DNA molecule.

This invention provides a method of determining physiological effects of expressing varying levels of HB9 protein in a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman animals, each a transgenic nonhuman mammal, each nonhuman mammal expressing

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a different amount of HB9 protein.

This invention provides a method of producing an isolated
purified HB9 protein which comprises: a) inserting a nucleic
5   acid molecule encoding a HB9 protein into a suitable vector;
b) introducing the resulting vector into a suitable host
cell; c) selecting the introduced host cell for the
expression of the HB9 protein; d) culturing the selected
cell to produce the HB9 protein; and e) recovering the HB9
10   protein produced.

This invention provides a method of inducing differentiation
of somatic motor neurons in a subject comprising
administering to the subject the purified HB9 protein in an
15   amount effective to induce differentiation of somatic motor
neurons in the subject.

This invention provides a pharmaceutical composition
comprising a purified HB9 protein and a pharmaceutically
20   acceptable carrier.

This invention provides a method for treating a subject
afflicted with an abnormality associated with a lack of one
or more normally functioning motor neurons which comprises
25   introducing an amount of a pharmaceutical composition
comprising a purified HB9 protein and a pharmaceutically
acceptable carrier effective to generate somatic motor
neurons from undifferentiated motor neuron precursor cells
in the subject, thereby treating the subject afflicted with
30   the abnormality associated with the lack of one or more
normally functioning motor neurons.

This invention provides a method of treating a subject
afflicted with a neurodegenerative disease which comprises
35   introducing an amount of a pharmaceutical composition which
comprises a purified HB9 protein and a pharmaceutically
acceptable carrier effective to generate somatic motor
neurons from undifferentiated precursor motor neuron cells

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in the subject, thereby treating the subject afflicted with
the neurodegenerative disease.

5   This invention provides for a method of treating a subject
         afflicted with an acute nervous system injury which
         comprises introducing an amount of a pharmaceutical
         composition which comprises a purified HB9 protein and a
         pharmaceutically acceptable carrier effective to generate
         motor neurons from undifferentiated precursor motor neuron
10   cells in a subject, thereby treating the subject afflicted
         with the acute nervous system injury.

         This invention provides for a method of treating a subject
         afflicted with an acute nervous system injury, wherein the
15   acute nervous system injury is localized to a specific
         central axon which comprises surgical implantation of a
         pharmaceutical composition comprising a HB9 protein and a
         pharmaceutically acceptable carrier effective to generate
         motor neurons from undifferentiated precursor motor neuron
20   cells located proximal to the specific central axon, so as
         to alleviate the acute nervous system injury localized to a
         specific central axon, thereby treating the subject
         afflicted with the acute nervous system injury.

25   This invention provides a method for diagnosing a chronic
         neurodegenerative disease associated with the expression of
         a HB9 protein in a sample from a subject which comprises:
         (a) obtaining DNA from the sample of the subject suffering
         from the chronic neurodegenerative disease; (b) performing
30   a restriction digest of the DNA with a panel of restriction
         enzymes; (c) separating the resulting DNA fragments by size
         fractionation; (d) contacting the resulting DNA fragments
         with a nucleic acid probe capable of specifically
         hybridizing with a unique sequence included within the
35   sequence of a nucleic acid molecule encoding a HB9 protein,
         wherein the sequence of a nucleic acid molecule encoding a
         HB9 protein is linked at a specific break point to a
         specified nucleic acid sequence and labeled with a

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detectable marker; (e) detecting labeled bands which have hybridized to the nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a HB9 protein,

5   wherein the sequence of a nucleic acid molecule encoding a HB9 protein is linked at a specific break point to a specified nucleic acid sequence to create a unique band pattern specific to the DNA of subjects suffering from the chronic neurodegenerative disease; (f) preparing DNA

10   obtained from a sample of a subject for diagnosis by steps (a-e); and (g) comparing the detected band pattern specific to the DNA obtained from a sample of subjects suffering from the chronic neurodegenerative disease from step (e) and the DNA obtained from a sample of the subject for diagnosis from

15   step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the chronic neurodegenerative disease if the patterns are the same.

20   This invention provides a method for diagnosing a chronic neurodegenerative disease associated with the expression of a HB9 protein in a sample from a subject which comprises: (a) obtaining RNA from the sample of the subject suffering from chronic neurodegenerative disease; (b) separating the

25   RNA sample by size fractionation; (c) contacting the resulting RNA species with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a HB9 protein, wherein the sequence of a nucleic acid molecule

30   encoding a HB9 protein is linked at a specific break point to a specified nucleic acid sequence and labeled with a detectable marker; (d) detecting labeled bands which have hybridized to the RNA species to create a unique band pattern specific to the RNA of subjects suffering from the

35   chronic neurodegenerative disease; (e) preparing RNA obtained from a sample of a subject for diagnosis by steps (a-d); and (f) comparing the detected band pattern specific to the RNA obtained from a sample of subjects suffering from

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the chronic neurodegenerative disease from step (d) and the RNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the chronic neurodegenerative disease if the patterns are the same.

This invention provides a functionally equivalent analog of HB9 that induces HB9 differentiation of neural progenitor cells.

This invention provides a functionally equivalent analog of HB9 that prevents HB9 differentiation of neural progenitor cells.

This invention provides a method of treating a subject afflicted with a neuromuscular disease which comprises introducing an amount of a pharmaceutical composition comprising a purified HB9 protein and a pharmaceutically acceptable carrier effective to activate acetylcholine to activate muscle cells.

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Brief Description of the Figures

Figures 1 A-H.

Isolation and Characterization of MNR2.

5

Figure 1A.

Hybridization analysis of cells isolated from [i] explants grown alone or with Shh. Left hand panels show cells isolated from explants grown with 2nM Shh and right hand

10   panels show cells isolated from explants grown without Shh. Top panels: ethidium labeling of PCR-amplified single cell-derived cDNAs. Lower panels: specific PCR-amplified transcripts in individual cells. Red circle indicates a motor neuron defined by Isl1, HB9 and Isl2 expression. Gray

15   circles indicate interneurons, defined by Lim2 expression.

Figure 1B.

Sequence similarity of the chick and human HB9 proteins (black line) and chick MNR2 and HB9 proteins (red line).

20   Conservation is indicated by higher similarity scores, using the PileUp and PlotSimilarity programs, Wisconsin Package 9.1, Genetics Computer Group. Chick MNR2 is 56% identical to chick HB9. Chick HB9 is 77% identical to human HB9. HD: homeodomain.

25

Figures 1C and 1D.

MNR2 expression in sections of cervical spinal cord of stage 12 **Fig. 1C.** and stage 16 **Fig. 1D.** embryos.

30   **Figure 1E.**

MNR2 expression in a stage 20 embryo. White circle shows position of the otic vesicle. Arrow indicates expression of MNR2 in abducens motor neurons.

35   **Figures 1F-H.**

Induction of MNR2 expression by Shh. [i] explants grown alone for 24h do not give rise to MNR2$^+$ cells **Fig. 1F.** [i] explants grown with Shh (4nM) for 24h contain many MNR2$^+$

cells **Fig 1G** not all of which express Isl1/2 **Fig 1H**. Images
representative of 4 explants.

Figures 2 A-K.
Expression of MNR2 Precedes that of Other Homeodomain
Proteins.

Figure 2A.
MNR2 expression (red cells) in the ventral neural tube of a
stage 18 embryo labeled with a pulse of BrdU (green cells).
Many cells coexpress MNR2 and BrdU (yellow cells).

Figure 2B.
Adjacent section to **Fig 2A.** showing that Isl1/2$^+$ cells (red
cells) do not coexpress BrdU (green cells).

Figure 2C.
Coexpression of MNR2 and MPM2 (Westendorf et al., 1994) in
a cell (yellow; arrow) in the ventral neural tube.
Approximately one MNR2$^+$, MPM2 cell was detected/10 μm
section, analysis of 300 MNR2$^+$ cells in 30 sections.

Figure 2D
Coexpression of MNR2 (red) and Pax6 (green) by ventral cells
in stage 20 spinal cord.

Figure 2E.
MNR2$^+$ cells (red) located dorsal to Nkx2.2$^+$ cells (green) in
stage 20 chick spinal cord.

Figure 2F.
MNR2 expression (red) in the caudal hindbrain (r7/r8) is
restricted to dorsal hypoglossal (somatic) motor neurons.
Ventral vagal (visceral) motor neurons express Isl1 (green)
but not MNR2.   MNR2 is absent from other cranial motor
neurons and from thoracic visceral motor neurons. MNR2
expression is excluded from oculomotor and trochlear motor
neurons, consistent with their expression of the visceral

motor neuron markers <u>Phox2a</u> and <u>Phox2b</u> (Pattyn et al.,
1997).

Figures 2G-J.

5   Expression of MNR2 (red) and other homeodomain protein
markers (green) of spinal motor neurons in stage 20 embryos.
Arrow in **Fig. 2G.** indicates dorsal Isl1$^+$ (D2) neurons. Lim3
$^+$ cells dorsal to MNR2$^+$ cells in **Fig. 2J.** are V2 neurons
(arrow).

10

Figure 2K.

Temporal sequence of homeodomain protein expression by
somatic motor neuron progenitors and newly-differentiated
somatic motor neurons. D2 and V2 neuron domains are shown.

15   Dotted line indicates cell cycle exit.

Figures 3A-J.

MNR2 Induces Somatic Motor Neuron Transcription Factors.

20   **Figure 3A.**

Sections of the spinal cord of an <u>MNR2</u>-infected embryo at
stage 23.   In this embryo, ectopic MNR2 expression is
detected predominantly on the right side.

25   **Figures 3B-E.**

Ectopic expression of Isl1 **Fig. 3B.** Isl2 **Fig. 3C.** HB9 **Fig.
3D.** and Lim3 **Fig. 3E.** in an <u>MNR2</u>-infected embryo. Ectopic
cells exhibit no dorsoventral (DV) restriction.   The
increase in the number of dorsal Isl1$^+$ cells **Fig 3B.** is not

30   caused by the precocious differentiation of D2 neurons (data
not shown).

Figure 3F

Detail of an <u>MNR2</u>-infected spinal cord showing that ectopic

35   Isl1$^+$ cells coexpress MNR2. Similar findings were obtained
for Isl2$^+$ and HB9$^+$ cells.

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Figure 3G.

Section through the spinal cord of an <u>MNR2</u>-infected embryo showing Pax7$^+$ dorsal progenitors (green). Ectopic MNR2 (red) does not repress Pax7 (green). Lateral MNR2$^+$, Pax7$^-$ cells are
5   post-mitotic neurons. Arrowhead indicates DV boundary.

Figures 3H-J.

Induction of Isl1/2 in ventral progenitors by MNR2. [i] explants isolated from <u>MNR2</u>-infected embryos and grown in
10   vitro for 24h with 0.5nM Shh contain many MNR2$^+$ cells **Fig. 3H.** Many of these cells express Isl1/2 **Fig. 3I.** [i] explants isolated from uninfected embryos and exposed to 0.5nM Shh do not contain MNR2$^+$ cells **Fig. 3J.** and do not give rise to Isl1/2$^+$ neurons (data not shown). Similar results obtained in
15   4 explants.

Figures 4A-J.

MNR2 Functions Within the Context of a General Neurogenic Program.

20

Figure 4A.

Coexpression of MNR2 (red) and Cyn1 (green) in the spinal cord of an <u>MNR2</u>-infected embryo, analyzed at stage 21. Cyn1$^+$, MNR2$^+$ cells are restricted to the lateral margin of the
25   spinal cord. MNR2 expression does not alter the number of Cyn1$^+$ cells.

Figure 4B.

Quantitative analysis of Cyn1 expression by ectopic MNR2$^+$
30   cells. Similar values were obtained from stages 20-23. Analysis derived from >300 MNR2$^+$ cells.

Figure 4C.

Detail of the dorsal spinal cord of an <u>MNR2</u>-infected embryo.
35   All ectopic Isl1/2$^+$ cells coexpress Cyn1 (green).

Figure 4D.

MNR2-induced ectopic Isl1$^+$ cells coexpress Cyn1. Analysis of

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>200 ectopic Isl1⁺ cells.

Figure 4E.

Proportion of MNR2-induced ectopic Isl2⁺, HB9⁺ and Lim3⁺ cells

5   that coexpress Isl1. All Isl2 ⁺ and HB9 ⁺ cells coexpress
Isl1. Only 55% of ectopic Lim3⁺ cells coexpress Isl1.
Analysis of 77 Isl2⁺, 38 HB9⁺ and 96 Lim3⁺ cells.

Figure 4F.

10   Many ectopic Lim3⁺ cells (red) in <u>MNR2</u>-infected embryos are
labeled (arrows) by a BrdU pulse (green).

Figure 4G.

Detail showing that many <u>MNR2</u>-induced ectopic Lim3⁺ cells

15   (green) do not express Isl1 (red).

Figure 4H.

Expression of homeodomain protein markers in ectopic MNR2⁺
neurons (MNR2⁺/Cyn1⁺ cells). Analysis of 690 Isl1 , 70

20   Isl2⁺, 111 HB9 ⁺ and 540 Lim3 ⁺ cells. More than 2000 MNR2 ⁺
cells analyzed from more than 40 <u>MNR2</u>-infected embryos.

Figure 4I.

Misexpression of <u>MNR2</u> in an embryo infected with a <u>MNR2</u> 3'Δ

25   construct. Section labeled with a 5' coding probe.

Figure 4J.

Activation of endogenous <u>MNR2</u> in an <u>MNR2</u>-infected embryo
revealed using a 3' non-coding probe. The endogenous <u>MNR2</u>

30   gene is activated both in neural and surrounding tissues.

Figures 5 A-J.

MNR2 Induces Later Features of the Somatic Motor Neuron
Phenotype.

35

Figures 5A-D.

<u>ChAT</u> expression in the spinal cord of uninfected **Fig. 5A.**
and **Fig.** 5C. and <u>MNR2</u>-infected embryos **Figs. 5B. and D.**

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Ectopic clusters of ChAT$^+$ cells are detected in the dorsal
spinal cord of MNR2-infected embryos (arrows in **Figs. 5 B**
and **D**).

5   **Figure 5E.**

Quantitation of ectopic dorsal ChAT$^+$ cell clusters in
uninfected and MNR2-infected embryos. Number of ectopic
dorsal ChAT$^+$ cell clusters detected in 70, 15 μm sections.

10   **Figures 5F-H.**

FITC-Dextran$^+$ (FITC-Dx) labeled neurons in the spinal cord of
stage 25 embryos after application of FITC-Dx to the ventral
roots. In uninfected embryos **Fig. 5F.**, retrogradely labeled
cells (green) are restricted to Isl1/2$^+$ motor neurons (red).

15   In MNR2-infected embryos ectopic FITC-Dx$^+$ Isl1/2 are detected
in the V1 and V2 neuron domain **Fig. 5G.** Ectopic FITC-Dx$^+$
neurons are also located in the dorsal spinal cord and
coexpress Isl1/2 **Fig. 5H** and MNR2 **Fig. 5I.** The axons of
these neurons project ventrally (arrows in **Fig. 5I**). Sensory

20   axons in the dorsal root entry zone have also been labeled
in some embryos (see **Fig. 5I**).

      Figure 5J.

Ectopic FITC-Dx$^+$ neurons in the dorsal spinal cord (DV

25   boundary is shown by dotted line in **Fig. 5H.**, analyzed in
80, 10 μm sections of uninfected and MNR2-infected embryos.
Ectopic FITC-Dx neurons in the V1 and V2 neuron domain are
not plotted in **Fig. 5J.**

30   **Figures 6A-P.**

Cooperation of MNR2 and Isl1 in the Induction of Somatic
Motor Neurons.

      Figures 6A-E.

35   Sections of the spinal cord of an embryo infected with Isl1
virus and analyzed at stage 20. Despite ectopic expression
of Isl1 **Fig. 6A.**, no ectopic MNR2$^+$ **Fig. 6B.**, Lim3$^+$ **Fig. 6C.**,
Isl2$^+$ **Fig. 6D.** or HB9$^+$ **Fig. 6E.** cells are detected. Images

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representative of six infected embryos.

Figs. 6F-H. Isl1, Isl2 and HB9 expression in an <u>MNR2</u>-
infected embryo analyzed at stage 20. Most Isl1⁺ neurons are
focused on the D2 neuron domain although more ventrally
5   located neurons are also detected **Fig. 6F**. All Isl2⁺ cells
Fig. 6G. coexpress Isl1 **Fig. 6H.** (see panel **Fig. 6P.** for
quantitation).

Figures. 6I, 6J.
10   HB9 expression in a stage 20 <u>MNR2</u>-infected embryo. All
ectopic HB9⁺ cells **Fig. 6I.** coexpress Isl1 **Fig. 6J.** See
panel **Fig 6P.** for quantitation.

Figures 6K-M.
15   Isl2 and HB9 expression in the spinal cord of an embryo
coinfected with <u>MNR2</u> and <u>Isl1</u> viruses, analyzed at stage 20.

Figure 6K.
Many ectopic MNR2⁺ cells (red) coexpress Isl1 (green) and
20   thus appear as yellow cells.

Figure 6L.
Ectopic expression of Isl2 in the spinal cord of an embryo
coinfected with <u>MNR2</u> and <u>Isl1</u> viruses, analyzed at stage 20.
25

Figure 6M.
Ectopic expression of HB9 in the spinal cord of an embryo
coinfected with <u>MNR2</u> and <u>Isl1</u> viruses, analyzed at stage 20.
Figure 6N.
30   Expression of Lim3, Isl2 and HB9 in ectopic MNR2⁺, Isl1⁺
cells. Analysis of more than 300 cells for each marker,
from four infected embryos.

Figures 6O. and P.
35   Distribution of ectopic Isl1 ⁺, Isl2⁺ and HB9⁺, cells along
the DV axis of the spinal cord of uninfected, <u>MNR2</u>-infected,
and <u>MNR2</u>/<u>Isl1</u>-coinfected embryos, analyzed at stage 20.
Diagram in **Fig. 6O.** shows the DV divisions (bins A-F) of the

spinal cord used to compile histograms shown in **Fig 6P**. Bins B and C normally contain Isl1$^+$ D2 neurons and bin F contains V2 neurons. Values indicate number of ectopic cells, mean $\pm$ SEM > 300 cells in 30 sections for each
5   marker.

Figure 7.
Cooperation of Lim3 and Isl1 as Mediators of MNR2 Activity and Mimicry by HB9.

10

Figure 7A.
Ectopic expression of Lim3 in the spinal cord of a <u>Lim3</u>-infected embryo, analyzed at stage 23.

15

Figure 7B.
Absence of ectopic MNR2$^+$ cells in the spinal cord of a <u>Lim3</u>-infected embryo. Analysis of more than 4000 ectopic Lim3$^+$ cells.

20

Figure 7C.
Absence of ectopic Isl2$^+$ cells in the spinal cord of a <u>Lim3</u>-infected embryo. Analysis of more than 5000 ectopic Lim3$^+$ cells.

25

Figure 7D.
Low incidence of ectopic expression of HB9 (green) in the spinal cord of a <u>Lim3</u>-infected embryo. All ectopic HB9 cells coexpress Isl1 (red). Analysis of more than 5000
30   ectopic Lim3$^+$ cells.

Figure 7E.
Quantitation of ectopic Isl1$^+$, Isl2$^+$ and HB9$^+$ cells in the dorsal spinal cord of a <u>Lim3</u>-infected embryo. Analysis of
35   300-600 Lim3$^+$ cells.

Figure 7F.
Ectopic expression of Chx10 (green) in the spinal cord of a

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Lim3-infected embryo. All ectopic Chx10⁺ cells coexpress
Lim3 (red) and thus appear as yellow cells.

Figure 7G.

Same image as in **Fig. 7F**, showing that ectopic Chx10⁺ cells
(green) are located both dorsal and ventral (arrowheads) to
the position of Isl⁺ motor neurons.

Figure 7H.

Coexpression of Isl1 (red) and Lim3 (green) in the spinal
cord of a Lim3/Isl1 coinfected embryo.

Figure 7I.

Isl2 expression is not detected in the spinal cord of a
Lim3/Isl1 coinfected embryo.

Figure 7J.

A high incidence of ectopic HB9 expression is detected in
the spinal cord of a Lim3/Isl1 coinfected embryo.

Figure 7K.

Ectopic Isl12 and HB9 expression in the dorsal spinal cord
of Lim3/Isl1 infected embryos. Analysis of 300-600 neurons
from six infected embryos.

Figure 7L.

Expression of HB9 in the spinal cord of an HB9-infected
embryo analyzed at stage 23.

Figure 7M.

Ectopic dorsal expression of Isl2 in the spinal cord of an
HB9-infected embryo. Analysis from >10 infected embryos.

Figure 7N.

Ectopic dorsal expression of Lim3 in the spinal cord of an
HB9-infected embryo.

Figure 8.

Role of MNR2 in the Shh-Induced Pathway of Somatic Motor

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Neuron and V2 Interneuron Generation.

In this model, homeodomain proteins (MNR2, Isl2, HB9) shown in red are restricted to the somatic motor neuron lineage and those (Lim 3, Chx10, V2 Interneuron) in blue to the V2
5   interneuron lineage. Vertical gray bar indicates time of exit from the cell cycle. The diagram indicates the subordinate activities of Lim3 and Isl1 in the activation of HB9, the requirement for Isl1 in the induction of HB9 and Isl2, and the autoactivation of MNR2. In the absence of
10   MNR2 activity, Lim3 is sufficient to induce Chx10. For details see the text.

FIGURE 9
Suppression of Spinal Interneuron Fates by MNR2.
15

Figure 9A.
Schematic diagram of the position of D1 (LH2$^+$), D2 (Isl1$^+$), V1 (En1$^+$) and V2 (Chx10$^+$) interneurons in the spinal cord of a stage 20-22 chick embryo.
20

Figure 9B.
Section through the spinal cord of a stage 22 <u>MNR2</u>-infected embryo showing the asymmetric distribution of ectopic MNR2$^+$ cells, which are restricted almost exclusively to the right
25   half of the spinal cord of this embryo.

Figures 9C-N.
Sections through the same embryo shown in **Fig. 9B.** double or triple-labeled to reveal ectopic Isl1$^+$ neurons and the
30   expression of D1, D2, V1 and V2 interneuron markers.

Figures 9C-E.
Shows the reduction (approximately 75%) in LH2 expression in the right half of the infected embryo.
35

Figures 9F-H.
Shows the reduction in Brn 3.0 expression (88 $\pm$ 4%, n = 4 sections) in the right half of the infected spinal cord.

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Figures 9I-K.

Shows the reduction in En1 expression (46 ± 5%; n = 10
sections) in the right half of the infected spinal cord.

Figures 9L-N.

Shows the reduction in Chx10 expression (89 ± 3%; n = 9
sections) in the right half of the infected spinal cord. A
similar repression of interneuron marker expression was
detected in six other MNR2-infected embryos.

Figure 10

cMNR2 Protein and the Predicted Amino Acid Sequence (SEQ ID
NO: 1).

Figure 11

cMNR2 DNA Nucleotides 1 -1736. (SEQ ID NO: 2).

Figure 12

cHB9 Protein and the Predicted Amino Acid Sequence (SEQ ID
NO: 3).

Figure 13

cHB9 DNA Nucleotides 1 -1534. (SEQ ID NO: 4).

**Figure 14. Early Expression of HB9 by Developing Motor
Neurons**

(A-D) Comparison of the expression of HB9 (red) and Lim3
(Lhx3) (green) in developing motor neurons at caudal
cervical levels of e9.5 to e10.0 mouse embryos. Percentage
numbers in each panel indicate the approximate fraction of
motor neurons detected at the time of analysis, compared to
the total number of motor neurons generated by e11.0 (see
Figure 3A). (A) At e9.5 (10-25% motor neuron generation),
HB9 and Lim3 are coexpressed by most labeled cells although
occasional Lim3$^+$/HB9$^-$ cells are detected. (B, C) At slightly
older ages (e9.75: 30-60% motor neuron generation), many
medially located Lim3$^+$/HB9$^-$ cells are detected and most
double-labelled cells are confined to the lateral margins of

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the ventral spinal cord.   (D) A similar profile is observed near the end of the period of motor neuron generation.   The notochord is also labelled at these developmental stages.

5   (E-H) Comparison of expression of HB9 (red) and Isl1 (green) in developing motor neurons located at caudal cervical levels of e9.5 to e10.0 mouse embryos. (E). In e9.5 embryos (10-15% motor neuron generation), HB9 and Isl1 are coexpressed by most cells, although occasional HB9$^+$/Isl1$^-$

10   cells are detected (arrows). (F, G) At slightly older stages (e9.75: 50-60% motor neuron generation) HB9 and Isl1 are coexpressed by virtually all cells in the ventral spinal cord. Note the expression of Isl1 but not of HB9 in sensory neurons in the dorsal root ganglia in (G).   A similar

15   profile is observed near the completion (85%) of motor neuron generation (H)

(I-L) Comparison of HB9 and MPM2 expression in developing motor neurons located at caudal cervical levels of e9.5 to

20   e10.0 mouse embryos. (I). In e9.5 embryos (10-15% motor neuron generation), 10% of medially located MPM2$^+$ cells express HB9 (arrow) although many ventral MPM2$^+$ cells within the domain of motor neuron generation lack HB9 expression. (J, K) At slightly older stages (e9.75) (25-60% motor neuron

25   generation) only 3% of MPM2 cells express HB9.   Near the completion of motor neuron generation (>60% motor neuron generation) no MPM2$^+$ cells coexpressed HB9, although many MPM2$^+$, Lim3$^+$ cells were detected (not shown).   MPM2 analysis was based on at least_6 embryos examined over the period

30   e9.5 to e10.0. (M) BrdU expression in many Lim3$^+$ cells in the ventral domain of motor neuron generation, assayed during the peak period (60%) of motor neuron genesis.

(N-P) Sections through e9.75 and e10.0 embryos pulse-labeled with BrdU in vivo for 2h. At early stages of motor neuron

35   genesis a few BrdU$^+$/HB9$^+$ cells are detected (N) but during the peak (O) and late (P) periods of motor neuron genesis, the number of BrdU$^+$/HB9$^+$ cells drops dramatically.   BrdU analysis was performed in at least 6 embryos.

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(Q) The position of generation of MNs and V2 interneurons. (R, S) Diagrams indicating the temporal profile of expression of HB9 in the early differentiation of mouse (R) and chick (S) spinal MNs, assessed by expression of homeodomain transcription factors. The sequential stages in the conversion of MN progenitors into post-mitotic MNs in chick are based on the data of Tanabe et al., 1998. Dashed vertical line indicates approximate time of cell cycle exit.

Figure 15. Inactivation of Hb9 by Homologous Recombination. (A) Diagrams show strategy for homologous recombination at the Hb9 locus in ES cells to generate a disrupted allele of Hb9 and the transgenic construct used to define a motor neuron regulatory region in the Hb9 locus. A 9kb NotI fragment comprising the 5' upstream region of the Hb9 gene and sequence from the first exon (blue) of Hb9 was used to generate a transgenic construct. To generate a disrupted Hb9 allele, targeting cassettes were integrated into the NotI site of the first of three exons of the Hb9 gene (data not shown). A 6kB 5' region (Sse83871-NotI) and a 3kB 3' region (NotI-XbaI) were used to generate the basic targeting vector into which the targeting cassettes (green) were integrated. The probe to screen ES cells for the detection of homologous recombination was a 1kB XbaI-XhoI (grey bar) fragment located 3' to the region used for the generation of the targeting construct.

(B) Targeting cassettes used for the generation of transgenic mice (i: IRES-nlslacZ) and generation of Hb9 alleles by homologous recombination in ES cells (ii-iv: ii: IRES-nlslacZ; iii: IRES-taulacZ; iv: IRES-taumyc). For homologous recombination, a PGK-NEO cassette flanked by loxP sites was inserted 3' to a SalI site . Right: Representative Southern blot of Hb9$^{nls-lacZ}$ genomic DNA derived from Hb9$^{+/+}$, Hb9$^{nlslacZ/+}$ and Hb9$^{nlslacZ/nlslacZ}$ embryos (6kB mutant band, 20kB wild type band; lines to the left indicate molecular weight standards; SalI/XhoI digest). Similar diagnostic blots were obtained from the other two targeted alleles (available on request).

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(C-F) Analysis of wholemount embryos stained for -
Galactosidase (-Gal) activity.

(C) e10 transgenic embryo (TgN(Hb9)SAX16), labeled for -Gal
expression.

(D) e11.5 Hb9<u>nlslacZ/+</u> embryo. The staining in the developing
limbs corresponds to the region of the zone of polarizing
activity (zpa).

(E, F) -Gal staining of e11.5 Hb9<u>taulacZ/+</u> (E) and compound
mutant Hb9<u>taulacZ/taumyc</u> (F) embryos. In the presence of one
allele of <u>tau-lacZ</u> the staining intensity in the compound
homozygote is five fold higher than in the heterozygote.
Embryos were processed for the same incubation time.

(F-H) Expression of HB9 in the spinal cord of e10.5 wild
type embryos (F). Coincidence of expression of HB9 protein
(green) and nls-lacZ (-Gal red) in e10.5 Hb9<u>nlslacZ/+</u> spinal
cord (G). Absence of HB9 protein in the spinal cord of
e10.5 Hb9<u>nlslacZ/nlslacZ</u> embryos (H).

Figure 16. Cell Migration Defects in <u>Hb9</u> Mutant Embryos.

(A) Analysis of number of -Gal labeled cells in brachial
spinal cord (cervical 7/8) at different developmental stages
(e10, e10.5, e12.5 and e13.5) in Hb9<u>nlslacZ/+</u> (solid line), and
Hb9<u>nlslacZ/nlslacZ</u> (dashed line) embryos.

(B-D) -Gal labeled neurons in Hb9<u>nlslacZ/nlslacZ</u> embryos are
located adjacent to the spinal cord within ventral roots.

(B) Whole mount -Gal staining of e10.5 lumbar spinal cord
showing extraspinal -Gal neurons.

(C) Extraspinal -Gal neurons (green) in an e12.5 embryo
coexpress Isl1/2 (red).

(D) Extraspinal -Gal cells (red) in an e12.5 embryo (arrow)
do not express the sensory neuron marker Brn3.0. The
position of the dorsal root ganglion (DRG) is shown.

(E-J) Organization of -Gal labeled cells in the CNS,
visualized in whole mount preparations of e17.5 Hb9<u>nlslacZ/+</u> (E,
G, I) and Hb9<u>nlslacZ/nlslacZ</u> (F, H, J) embryos.

(E, F) Lateral view of caudal hindbrain and cervical level
spinal cord. Arrows point to rostrocaudal level of
hypoglossal MNs. Note the clustering of the -Gal labeled

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cells in the hypoglossal nucleus in heterozygous embryos but the scattered organization of -Gal labeled cells along both the dorsoventral and rostrocaudal axes of homozygous Hb9<u>nlslacZ</u> embryos.

5   (G, H) Ventral view of forelimb level spinal cord. Note the clear segregation of the MMC (medial strip of -Gal labeled cells) and LMC (lateral group of -Gal labeled cells) in the spinal cord of heterozygous Hb9<u>nlslacZ</u> embryos (G) and the extensive intermixing of -Gal labeled cells in homozygous

10   Hb9<u>nlslacZ</u> embryos (H).

(I, J) Ventral view of lumbar level spinal cord. A clear segregation of -Gal labeled cells in MMC (medial strip) and LMC (lateral strip) is observed in the spinal cord of heterozygous Hb9<u>nlslacZ</u> embryos (I) but not in homozygous

15   Hb9<u>nlslacZ</u> embryos (J). Similar observations were made in younger stage embryos (e14.5 to e16.5).

Figure 17. Transient Deregulation of Lim3 in Motor Neurons in Mice Lacking Hb9.

20   Analysis of Lim3 and Isl1 expression in -Gal labeled MNs in brachial spinal cord (level C7/8) at different developmental stages in Hb9<u>nlslacZ/+</u> and H̶b̶9̶<u>nlslacZ/nlslacZ</u> embryos.

(A, B, E, F) Triple label immunocytochemical detection of Lim3 (green), -Gal (A, B: red) and Isl1 (E, F: red) on the

25   same sections (A, E and B, F) of e10.5 Hb9<u>nlslacZ/+</u> and Hb9<u>nlslacZ/nlslacZ</u> embryos. In H̶b̶9̶<u>nlslacZ/+</u> embryos, MMC MNs maintain Lim3 expression but most MNs rapidly downregulate Lim3. In Hb9<u>nls-lacZ/nls-lacZ</u> embryos examined at this stage, virtually all Isl1/ -Gal cells coexpress Lim3.

30   (C, D, G, H) Developmental time course of Lim3 and Isl1 expression in Hb9<u>nlslacZ/+</u> (solid lines) a̶n̶d̶ <u>nlslacZ/nlslacZ</u> (dashed lines) embryos. (C) Number of Lim3 cells. (D) Number of Lim3, -Gal cells. (G) Number of Isl1 cells. (H) Number of Lim3, Isl1 cells. Data from at least three independent

35   sets of experiments for each developmental stage (e10, e10.5, e12.5 and e13.5) are shown. For each stage the number of positive cells per ventral quadrant of the spinal cord was counted on at least four consecutive sections.

Figure 18. V2 Interneuron Marker Expression by Motor Neurons but no Accompanying Change in Axonal Trajectory in Mice Lacking Hb9.

Analysis of Chx10 expression by -Gal labeled cells and Isl1
5   MNs at different developmental stages in brachial spinal cord (level C7/8) of Hb9<u>nlslacZ/+</u> (**A, C, D, F**) and <u>Hb9nlslacZ/nlslacZ</u> (**B, C, E, F**) embryos.

(**A, B, D, E**) Triple label immunocytochemical analysis of Chx10 (green), -Gal (**A, B**: red) and Isl1 (**D, E**: red)
10   expression in sections of e10.5 spinal cord from Hb9<u>nlslacZ/+</u> (**A, D**) and Hb9<u>nlslacZ/nlslacZ</u> (**B, E**) embryos. In <u>Hb9nlslacZ/+</u> embryos, there is no coincidence of expression of -Gal or Isl1 with Chx10. In Hb9<u>nlslacZ/nlslacZ</u>, many Isl1 and -Gal labeled cells coexpress Chx10.

15   (**C, F**) Developmental time course of Chx10 expression in Hb9<u>nlslacZ/+</u> (solid lines) and
Hb9<u>nlslacZ/nlslacZ</u> (dashed lines) embryos. The peak number of Chx10, -Gal cells in Hb9<u>nlslacZ/nlslacZ</u> embryos occurred at e10.5 after which their number gradually declined to that observed
20   in Hb9<u>nlslacZ/+</u> embryos. Data from at least three independent sets of experiments are shown for each developmental stage (e10, e10.5, e12.5 and e13.5). For each stage the number of labeled cells per ventral quadrant of the spinal cord was counted on at least four consecutive sections.

25   (**G-N**) Retrograde HRP labeling from the base of the forelimb of e11.5 heterozygous Hb9<u>nlslacZ</u> (**G, I**) and homozygous <u>Hb9nlslacZ</u> (**H, J**) embryos. Triple label immunocytochemical detection of Chx10 (green), HRP (**G, H**: red) and Isl1 (**I, J**: red) on the same section (**G, I** and **H, J**). Arrows point to Chx10, HRP
30   colabeled cells, two of which do not express Isl1.

(**K-N**) Axonal projection of Hb9 neurons in Hb9<u>taumyc/+</u> (**K, M**) and Hb9<u>taumyc/taumyc</u> (**L, N**) embryos, detected using anti-Myc epitope antibodies.

(**K, L**) Transverse sections of e13.5 spinal cord show myc-
35   labeled axons projecting into the ventral roots.

(**M, N**) Parasagittal sections of e13.5 spinal cord (R: rostral, C: caudal, M: medial; L: lateral) showing MN cell bodies and intersegmental projections in the ventrolateral

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funiculus (vlf, white bar). Myc-labeled (red) axons are not
detected amongst the neurofilament (NF) 160-labeled (green)
axons in the vlf in Hb9 <u>taumyc/taumyc</u> embryos, indicating the
absence of intersegmental projections of ectopic Chox10

5   neurons. HRP injection into the vlf of the spinal cord of
e13.5 wild type embryos labels ipsilateral Chx10 neurons at
more caudal segmental levels (data not shown).

Figure 19. Defects in Motor Neuron Subtype Identity in Hb9
10   **Mutant Mice.**

(**A**) Diagram summarizing sequential steps in motor neuron
(MN) differentiation in the developing spinal cord. MNs
(red) initially acquire a generic identity that
distinguishes them from neighboring interneurons (in this

15   diagram only V2 interneurons are shown). MNs initially
express Isl1, whereas V2 neurons (blue) express Chx10. There
are two major classes of spinal MNs, visceral and somatic,
that project to different target cells. Most visceral MNs
can be identified by expression of NADPH-diaphorase (NADPH-

20   d) and somatic MNs by expression of Isl2. The somatic MN
class consists of two major columns, the median (MMC) and
lateral (LMC) motor columns that project to skeletal muscles
in different peripheral locations. LMC MNs can be identified
by expression of RALDH2 and MMC neurons by expression of

25   Lim3 and Gsh4. Within the LMC, neurons in the medial (m)
division coexpress Isl1 and Isl2 and project to ventrally-
derived limb muscles whereas neurons in the lateral (l)
division coexpress Isl2 and Lim1 and project to dorsally-
derived limb muscles. Within each LMC division, MN pools

30   that project to individual limb muscles can be identified by
expression of the ETS domain proteins PEA3 and ER81. For
details, see Tsuchida et al., 1994, Ericson et al., 1997a,
Sockanathan and Jessell, 1998, Lin et al., 1998 and the
text. (**B-O**) Expression of MN subtype markers in developing

35   spinal MNs. Images show e13.5 thoracic spinal cord (**B, C,F,
G**), e12.5 C7/8 level spinal cord (**D, E**), and e13.5 C7/8
level spinal cord (**H-O**) in Hb9<u>nlslacZ/+</u> and Hb9<u>nlslacZ/nlslacZ</u>
embryos.

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(**B, C**) <u>Choline Acetyltransferase</u> (<u>ChAT</u>, generic MN marker) expression detected by in situ hybridization. Arrows in (**B**) point to medial populations of visceral MNs.

(**D, E**) -Gal cells (red) coexpress Isl2 (green; a somatic MN-specific marker at this stage). The number of Isl2 cells is similar in $Hb9^{nlslacZ/+}$ and $Hb9^{nlslacZ/nlslacZ}$ embryos examined at e12.5. By e13.5, the number of Isl2 cells in mutants is, however, reduced by ~40% compared to heterozygote embryos (data not shown).

(**F, G**) NADPH-diaphorase enzyme activity in visceral MNs. In heterozygous $Hb9^{nlslacZ}$ embryos, both lateral (l) and medial (m) populations of NADPH diaphorase neurons are present (**F**). In homozygous $Hb9^{nlslacZ}$ embryos, NADPH-diaphorase expression is absent from medial regions of the intermediate zone (**G**) although many cells in this region still express lacZ (data not shown).

(**H, I**) Coexpression of Lim3 (green) and -Gal (red) delineates the medial MMC in heterozygous $Hb9^{nlslacZ}$ embryos (**H**). Lim3, -Gal cells in homozygous $Hb9^{nlslacZ}$ embryos are not restricted to the region of the medial MMC (**I**).

(**J, K**) In situ hybridization analysis of <u>RALDH2</u> expression delineates LMC neurons (**J**). There is a marked reduction of <u>RALDH2</u> expression in homozygous $Hb9^{nlslacZ}$ embryos (**K**).

(**L, M**) Coexpression of Isl2 (red) and Lim1/2 (green) delineates lateral LMC neurons (**L**). There is a marked reduction of Isl2, Lim1 cells in homozygous $Hb9^{nlslacZ}$ embryos (**M**).

(**N, O**) PEA3 expression (green; motor pool marker) by pectoralis MNs (**N**) is severely reduced in homozygous $Hb9^{nlslacZ}$ embryos (**O**). PEA3 expression by DRG neurons (arrows) is not affected in homozygous $Hb9^{nlslacZ}$ embryos.

Figure 20. Defects in Motor Axon Projections in <u>Hb9</u> Mutant Mice.

(**A-D**) Whole mount -Gal staining of $Hb9^{taulacZ}$ embryos.

(**A-C**) Perturbation in motor axon projections in e11.5 $Hb9^{taulacZ/taulacZ}$ embryos (**B, C**), when compared with the axonal

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projection pattern observed in Hb9<u>taulacZ/+</u>embryos (**A**). Arrows point to the hypoglossal nerve which is absent or misrouted in some mutants (**B**) but present (**C**) in others. Asterisk in C indicates expanded plexus region at the base of the forelimb.

(**D**) Dorsal (d) and ventral (v) motor axon branches in the hindlimb of a e12.5 Hb9<u>taulacZ/taulacZ</u> embryo. Note -Gal labeling in the zone of polarizing activity (zpa) of the developing limb.

(**E-H**) Analysis of peripheral projections of motor axons in sections of e12.5 Hb9<u>taumyc/+</u> (**E**, **F**) and ~~Hb9<u>taumyc/taumyc</u>~~ (**G**, **H**) embryos. Double label immunocytochemical detection of myc-labeled (red) axons projecting towards skeletal muscles (-actinin-labeled; green). Axial muscle nerve branches are indicated (arrows, a). Star indicates the expansion of the axial nerve branch point (**H**). The dorsally (d) and ventrally (v) directed branches of motor axons in the limb are evident in Hb9 heterozygous (**E**) and homozygous (**G**) mutant embryos

(**I-L**) Analysis of innervation of diaphragm muscle of post-natal day 0 wild type (**I**, **K**) and homozygous Hb9<u>nlslacZ</u> (**J**, **L**) mice.

(**I,J**) Whole-mount -bungarotoxin (-BTX) staining shows the presence of AChR clusters localized in a tight band in the diaphragm of wild type embryos (**I**) and a scattered distribution of AChR clusters over a larger region of the diaphragm muscle in homozygous Hb9<u>nlslacZ</u> embryos (**J**).

(**K, L**) Double label immunocytochemical detection of GAP43-labeled axons and nerve terminals (green) and -bungarotoxin-labeled AChR clusters (red). Transverse section through the diaphragm reveals the coincidence (yellow patches) of GAP43 and -bungarotoxin labeling in wild-type muscle (**K**) and the lack of coincidence of label in homozygous Hb9<u>nlslacZ</u> embryos (**L**).

Figure 21. Functions of HB9 in the Differentiation of Post-Mitotic Motor Neurons.
Proposed functions of HB9 in post-mitotic MNs. Red arrows

highlight the steps in MN differentiation that appear to be
controlled by HB9.   HB9 appears to have two main, and
possibly interrelated, functions.   First HB9 is normally
required for the rapid down-regulation of Lim3 (and Gsh4)
from most post-mitotic MNs, which in turn appears to prevent
expression of the V2 interneuron marker Chx10. One exception
to this function of HB9 is in the context of medial MMC
neurons, where Lim3 and Gsh4 expression persists in MNs for
a prolonged period, even in wild-type embryos (Tsuchida et
al., 1994; Sharma et al., 1998). Thus, medial MMC neurons
appear to be subject to distinct regulatory controls on the
timing of Lim3/Gsh4 expression and by inference, on the
suppression of V2 interneuron character.   Second, HB9 is
required for the maintenance of Isl1 expression in post-
mitotic MNs.   A negative autoregulatory activity of HB9 is
also shown and is indicated as a direct interaction,
although it may be mediated indirectly. Approximate timing
of cell cycle exit with respect to homeodomain protein
expression is indicated.   HB9 is also required, directly or
indirectly, for the efficient establishment of the class,
columnar, divisional and pool identities of spinal MNs.
Dashed lines indicate that the erosion of MN subtype
identity could reflect a direct action of HB9, the loss of
expression of proteins such as Isl1 that normally serve a
positive function in MN differentiation, or the ectopic
expression of interneuron markers such as Chx10, or from a
combination of these events.   Proteins indicated in grey
delineate markers of MN subtype identity that are affected
in Hb9 mutants. Shh : Sonic hedgehog, an inductive signal
required for motor neuron generation and HB9 expressoin.
For further details, see text.

Fig. 22. HB9 is transiently expressed in the early
developing pancreatic anlagen but reappears in insulin
producing -cells. (a-f) HB9 and IPF1/PDX1 expression in
pancreatic rudiments between stage e8-e10.5. (a) HB9 is
expressed in both dorsal and ventral pancreatic epithelia in
8-10 somites stage embryos (e8), whereas (b) IPF1/PDX1

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expression is only detected in the ventral pancreatic
epithelium at this stage. Note that HB9 is also expressed in
the notochord at this stage (indicated by the arrow). (c,d)
By e9.5, HB9 and IPF1/PDX1 expression can be detected in
5   both pancreatic buds although HB9 expression is expressed at
a low level. (e) In e10.5 embryos the ventral HB9 expression
is virtually absent and only very low levels of HB9
expression can be detected in the dorsal bud. (f) In
contrast, IPF1/PDX1 expression is still expressed in both
10   pancreatic buds. (g-i) Double immunohistochemical analysis
of e17.5 pancreas using anti-HB9 and anti-insulin (g), anti-
glucagon (h) or anti-somatostatin (i) antibodies. At this
stage HB9 expression is restricted to the insulin producing
cells. Abbreviations: d, dorsal pancreatic epithelium; v,
15   ventral pancreatic epithelium.

Fig. 23. Hb9 deficient mice lack the dorsal pancreas. (a,b)
The pancreatic region of e17.5 (a) and e13.5 (b) Hb9^{nlslacZ}
heterozyogous and homozygous littermates demonstrating the
20   loss of dorsal pancreatic epithelium whereas the ventral
pancreas, dorsal mesenchyme and spleen develops in
homozygous mutant embryos. (c-i) The development of the
dorsal pancreas is arrested before the stage of epithelial
evagination in Hb9^{nlslacZ} homozygous embryos. (c-i) X-gal
25   staining or IPF1/PDX1 immunohistochemistry of whole-mount
and transversal cryostat sections of e9.5-10.5 heterozygous
and homozygous embryos. (c,d) Whole-mount X-gal staining of
e9.5 embryos shows that the protruding, dorsal pancreatic
bud can be detected in the heterozygous embryos whereas no
30   equivalent structure is found in the homozygous embryos.
(e,f) Immunohistochemical analysis of heterozygous and
homozygous e9.5 embryos demonstrates the absence of
IPF1/PDX1 expression in the presumptive dorsal pancreatic
epithelium of homozygous embryos. (g-i) X-gal staining of
35   e10.5 embryos showing that at this stage the ventral bud has
formed in both the heterozygous and homozygous embryos
whereas there is no evidence of a dorsal pancreatic bud in
homozygous embryos. Note that lacZ activity still can be

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readily detected in both buds at this stage although, when
analysed immunohistochemically using anti-HB9 antibodies,
HB9 protein expression is barely detectable at this stage
(see Fig. 1e for comparison). This is probably due to a
5   combination of high sensitivity when staining for lacZ
activity as compared to using antibodies, together with the
persistence of -galactosidase protein. No lacZ activity is
detected in the notochord at this stage. Abbreviations: s,
stomach; d, duodenum; sp, spleen; dp, dorsal pancreas; vp,
10   ventral pancreas; MN, motor neurons. (Arrowhead indicates
dorsal pancreatic bud.)

Fig. 24. Early pancreatic cell differentiation of the dorsal
bud is impaired in the Hb9nlslacZ homozygotes. (a-f)
15   Immunohistochemical analyses of e9.5 wild-type (a,c,e) and
Hb9nlslacZ homozygous (b,d,f) embryos using anti-Isl-1 (a,b),
anti-Nkx2.2 antibodies (c,d) and anti-glucagon (e,f)
antibodies. None of these early pancreatic markers is
detected in the dorsal pancreatic epithelium. (g-j) Shh
20   (g,h) and Ihh (i,j) expression is unaffected in e9.5 Hb9nlslacZ
homozygotes (h,j) as compared to stage-matched wild-type
littermates (g,i). The broken line indicates the pancreatic
buds

25   Fig. 25. The ventral pancreas of Hb9 deficient mice exhibit
a perturbed islet cell organisation with immature -cells.
(a-j) Immunohistochemical analysis of pancreatic marker
expression in the ventral pancreas derived from a wild-type
(a,c,e,g,i) and Hb9nlslacZ homozygous (b,d,f,h,j) neonates.
30   Both endocrine and exocrine cells appear as shown by the
presence of (a-d,g,h) insulin⁺, (a,b) glucagon , (c,d)
somatostatin⁺, and (e,f) amylase⁺ cells but the endocrine
cells fail to organise themselves into the typical structure
of maturing islets. (g-j) The insulin-positive cells present
35   in the ventral bud of Hb9nlslacZ homozygous express IPF1/PDX1
but not Glut2 indicating that they are not terminally
differentiated.

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Detailed Description of the Invention

The following standard abbreviations are used throughout the
specification to indicate specific nucleotides:

5

C=cytosine
A=adenosine
T=thymidine
G=guanosine

10

This invention provides an isolated nucleic molecule
encoding a motor neuron restricted pattern, MNR2, protein.
In an embodiment the isolated nucleic molecule encoding a
motor neuron restricted pattern, MNR2, protein is a DNA
15   molecule. In another embodiment the isolated nucleic acid
molecule encoding a motor neuron restricted pattern, MNR2,
protein is a cDNA molecule. In a further embodiment the
isolated DNA molecule encoding a motor neuron restricted
pattern, MNR2, protein is a RNA molecule. In an embodiment
20   the isolated nucleic acid molecule encoding a motor neuron
restricted pattern is operatively linked to a promoter of
RNA transcription.

The DNA molecules of the subject invention also include DNA
25   molecules coding for polypeptide analogs, fragments or
derivatives of antigenic polypeptides which differ from
naturally-occurring forms in terms of the identity or
location of one or more amino acid residues (deletion
analogs containing less than all of the residues specified
30   for the protein, substitution analogs wherein one or more
residues specified are replaced by other residues and
addition analogs where in one or more amino acid residues is
added to a terminal or medial portion of the polypeptides)
and which share some or all properties of naturally-
35   occurring forms. These molecules include: the incorporation
of codons "preferred" for expression by selected non-
mammalian hosts; the provision of sites for cleavage by
restriction endonuclease enzymes; and the provision of

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additional initial, terminal or intermediate DNA sequences
that facilitate construction of readily expressed vectors.

5   The DNA molecules described and claimed herein are useful
        for the information which they provide concerning the amino
        acid sequence of the polypeptide, MNR2, and as products for
        the large scale synthesis of the polypeptide MNR2, or
        fragments thereof, by a variety of recombinant techniques.
10   The DNA molecule is useful for generating new cloning and
        expression vectors, transformed and transfected prokaryotic
        and eukaryotic host cells, and new and useful methods for
        cultured growth of such host cells capable of expression of
        the polypeptide MNR2 or portions thereof and related
15   products.

        In another embodiment the isolated nucleic acid molecule
        which is a cDNA molecule which, encoding a motor neuron
        restricted pattern MNR2 protein, encodes a chick MNR2
20   protein. In another embodiment the isolated nucleic acid
        molecule is a cDNA molecule wherein the nucleic acid
        molecule encodes a chick MNR2 protein comprising the amino
        acid sequence set forth in SEQ ID NO: 1. In a further
        embodiment the isolated nucleic acid molecule is a cDNA
25   molecule wherein the nucleic acid molecule encodes a
        mammalian MNR2 protein. In an embodiment the isolated
        nucleic acid molecule is a cDNA molecule wherein the nucleic
        acid molecule encodes a mammalian MNR2 protein which is a
        mouse, rat or human protein. In an embodiment the isolated
30   nucleic acid molecule is a cDNA molecule, which comprises
        the nucleic acid sequence set forth in SEQ ID NO: 2.

        This invention provides a vector which comprises the
        isolated nucleic acid encoding a motor neuron restricted
35   pattern, MNR2, protein operatively linked to a promoter of
        RNA transcription. In an embodiment a plasmid comprises the
        vector which comprises the isolated nucleic acid encoding a
        motor neuron restricted pattern, MNR2, protein. In another

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embodiment the plasmid, which comprises the vector which comprises the isolated nucleic acid encoding a chick motor neuron restricted pattern, pMNR2, protein, is designated pMNR2.

In an embodiment, a full-length cDNA nucleic acid molecule encoding a MNR2 protein is inserted into a pcs2$^+$ plasmid and the resulting plasmid is designated as pcs2$^+$MNR2. The plasmid is with ampicillin resistance and 1.2 kilobase insert is releasable by cleavage with ClaI restriction endonuclease. Plasmid pcs2$^+$MNR2 was deposited on September 28, 1998 with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. Plasmid pcs2$^+$MNR2 was accorded ATCC Accession Number 203294.

Numerous vectors for expressing the inventive proteins may be employed. Such vectors, including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses, are well known in the art. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow for the selection of transfected host cells. The markers may provide, for example, prototrophy to an auxotrophic host, biocide resistance or resistance to heavy metals such as copper. The selectable marker gene can be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription

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initiation sequences for ribosome binding. Additional
elements may also be needed for optimal synthesis of mRNA.
These additional elements may include splice signals, as
well as enhancers and termination signals. For example, a
5   bacterial expression vector includes a promoter such as the
lac promoter and for transcription initiation the Shine-
Dalgarno sequence and the start codon AUG. Similarly, a
eukaryotic expression vector includes a heterologous or
homologous promoter for RNA polymerase II, a downstream
10   polyadenylation signal, the start codon AUG, and a
termination codon for detachment of the ribosome. Such
vectors may be obtained commercially or assembled from the
sequences described by methods well known in the art, for
example the methods described above for constructing vectors
15   in general.

These vectors may be introduced into a suitable host cell to
form a host vector system for producing the inventive
proteins. Methods of making host vector systems are well
20   known to those skilled in the art.

Methods of introducing nucleic acid molecules into cells are
well known to those of skill in the art. Such methods
include, for example, the use of viral vectors and calcium
25   phosphate co-precipitation.

The "suitable host cell" in which the nucleic acid molecule
encoding is MNR2 protein capable of being expressed is any
cell capable of taking up the nucleic acid molecule and
30   stably expressing the MNR2 encoded thereby.

This invention provides a host cell containing the vector
which comprises the isolated nucleic acid encoding a motor
neuron restricted pattern, MNR2, protein. In an embodiment
35   the host cell is selected from a group consisting of a
bacterial cell, a plant cell, an insect cell and a mammalian
cell.

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Suitable host cells include, but are not limited to, bacterial cells (including gram positive cells), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to HeLa cells, Cos cells, CV1 cells and various primary mammalian cells. Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH-3T3 cells, CHO cells, HeLa cells, Ltk⁻ cells and COS cells. Mammalian cells may be transfected by methods well known in the art such as calcium phosphate precipitation, electroporation and microinjection.

This invention provides a method of producing a polypeptide having the biological activity of a mammalian MNR2 which comprises growing host cells selected from a group consisting of bacterial, plant, insect or mammalian cell, under suitable conditions permitting production of the polypeptide. In another embodiment of the method of producing a polypeptide having the biological activity of a mammalian MNR2 the method further comprises of the recovering the produced polypeptide.

This invention provides an isolated nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the nucleic acid molecule encoding a motor neuron restricted pattern, MNR2, protein. In an embodiment the isolated nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the nucleic acid molecule encoding a motor neuron restricted pattern, MNR2, protein is a DNA molecule. In another embodiment the isolated nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the nucleic acid molecule encoding a motor neuron restricted pattern, MNR2, protein, is a RNA molecule.

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This invention provides an isolated nucleic acid molecule capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule which is complementary to the nucleic acid molecule encoding

5   a motor neuron restricted pattern, MNR2, protein. In an embodiment the isolated nucleic acid molecule capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule which is complementary to the nucleic acid molecule encoding a motor

10   neuron restricted pattern, MNR2, protein, is a DNA molecule. In another embodiment the isolated nucleic acid molecule capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule which is complementary to the nucleic acid molecule encoding

15   a motor neuron restricted pattern, MNR2, protein is a RNA molecule.

One of ordinary skill in the art will easily obtain unique sequences from the cDNA cloned in the pMNR2 plasmid. Such

20   unique sequences may be used as probes to screen various mammalian cDNA libraries and genomic DNAs, e.g. mouse, rat and bovine, to obtain homologous nucleic acid sequences and to screen different cDNA tissue libraries to obtain isoforms of the obtained nucleic acid sequences. Nucleic acid probes

25   from the cDNA cloned in the pMNR2 plasmid may further be used to screen other human tissue cDNA libraries to obtain isoforms of the nucleic acid sequences encoding MNR2 protein as well as to screen human genomic DNA to obtain the analogous nucleic acid sequences. The homologous nucleic

30   acid sequences and isoforms may be used to produce the proteins encoded thereby.

As used herein, "capable of specifically hybridizing" means capable of binding to an mRNA molecule encoding a MNR2 but

35   not capable of binding to an mRNA molecule encoding a MNR2 receptor protein.

This invention provides an antisense oligonucleotide having

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a nucleic acid sequence capable of specifically hybridizing to an mRNA molecule encoding a MNR2 protein. In an embodiment the antisense oligonucleotide has a nucleic acid sequence capable of specifically hybridizing to the isolated cDNA molecule encoding a motor neuron restricted pattern, MNR2, protein. In an embodiment the antisense oligonucleotide has a nucleic acid sequence capable of specifically hybridizing to an isolated RNA molecule encoding a motor neuron restricted pattern, MNR2 protein.

This invention provides a purified MNR2 protein. In an embodiment the purified MNR2 protein is encoded by an isolated nucleic acid encoding a motor neuron restricted pattern, MNR2, protein. In another embodiment the MNR2 protein unique polypeptide fragment of the purified MNR2 protein. In an embodiment the purified MNR2 protein has substantially the same amino acid sequence as set forth in SEQ ID NO: 1. In a further embodiment the purified MNR2 protein having an amino acid sequence as set forth in SEQ ID NO: 1. In another embodiment the purified MNR2 protein has an amino acid sequence as set forth in SEQ ID NO: 1. In a further embodiment, the MNR2 protein is a vertebrate MNR2 protein. In an embodiment the purified vertebrate MNR2 protein having an amino acid sequence as set forth in SEQ ID NO: 12 is a chick, mouse or rat MNR2 protein.

As used herein, an MNR2 protein having "substantially the same" amino acid sequences as set forth in SEQ ID NO: 1 is encoded by a nucleic acid encoding MNR2, said nucleic acid having 100% identity in the homeodomain regions, that is those regions coding the protein, and said nucleic acid may vary in the nucleotides in the non-coding regions.

This invention provides a monoclonal antibody directed to an epitope of an MNR2 protein. In an embodiment the monoclonal antibody is directed to a chick, mouse or rat MNR2 protein.

This invention provides a polyclonal antibody directed to an

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epitope of the purified MNR2 protein having the amino
sequence as set forth in SEQ ID No: 1.   In a further
embodiment the monoclonal or polyclonal antibodies are
directed to the MNR2 protein, having the amino sequence as
5   set forth in SEQ ID NO: 1.

Polyclonal antibodies may be produced by injecting a host
animal such as rabbit, rat, goat, mouse or other animal with
the immunogen of this invention, e.g. a purified mammalian
10   MNR2 protein or a purified human MNR2 protein. The sera are
extracted from the host animal and are screened to obtain
polyclonal antibodies which are specific to the immunogen.
Methods of screening for polyclonal antibodies are well
known to those of ordinary skill in the art such as those
15   disclosed in Harlow & Lane, Antibodies: A Laboratory Manual,
(Cold Spring Harbor Laboratories, Cold Spring Harbor, NY:
1988) the contents of which are hereby incorporated by
reference.

20   The monoclonal antibodies may be produced by immunizing for
example, mice with an immunogen. The mice are inoculated
intraperitoneally with an immunogenic amount of the above-
described immunogen and then boosted with similar amounts of
the immunogen. Spleens are collected from the immunized mice
25   a few days after the final boost and a cell suspension is
prepared from the spleens for use in the fusion.

Hybridomas may be prepared from the splenocytes and a murine
tumor partner using the general somatic cell hybridization
30   technique of Kohler, B. and Milstein, C., Nature (1975) 256:
495-497. Available murine myeloma lines, such as those from
the American Type Culture Collection (ATCC) 12301 Parklawn
Drive, Rockville, MD 20852 USA, may be used in the
hybridization. Basically, the technique involves fusing the
35   tumor cells and splenocytes using a fusogen such as
polyethylene glycol.   After the fusion the cells are
separated from the fusion medium and grown in a selective
growth medium, such as HAT medium, to eliminate unhybridized

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parent cells. The hybridomas may be expanded, if desired, and supernatants may be assayed by conventional immunoassay procedures, for example radioimmunoassay, using the immunizing agent as antigen. Positive clones may be

5   characterized further to determine whether they meet the criteria of the invention antibodies.

Hybridomas that produce such antibodies may be grown in vitro or in vivo using known procedures. The monoclonal

10   antibodies may be isolated from the culture media or body fluids, as the case may be, by conventional immunoglobulin purification procedures such as ammonium sulfate precipitation, gel electrophoresis, dialysis, chromatography, and ultrafiltration, if desired.

15

In the practice of the subject invention any of the above-described antibodies may be labeled with a detectable marker. In one embodiment, the labeled antibody is a purified labeled antibody. As used in the subject

20   invention, the term "antibody" includes, but is not limited to, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and binding fragments thereof. Furthermore, the term "antibody" includes chimeric

25   antibodies and wholly synthetic antibodies, and fragments thereof.

Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments

30   thereof. A "detectable moiety" which functions as detectable labels are well known to those of ordinary skill in the art and include, but are not limited to, a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected

35   through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase, ß-galactosidase, fluorescein or steptavidin/biotin. Methods

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of labeling antibodies are well known in the art.

Determining whether the antibody forms such a complex may be accomplished according to methods well known to those
5   skilled in the art.   In the preferred embodiment, the determining is accomplished according to flow cytometry methods.

The antibody may be bound to an insoluble matrix such as
10   that used in affinity chromatography.   As used in the subject invention, isolating the cells which form a complex with the immobilized monoclonal antibody may be achieved by standard methods well known to those skilled in the art. For example, isolating may comprise affinity chromatography
15   using immobilized antibody.

Alternatively, the antibody may be a free antibody. In this case, isolating may comprise cell sorting using free, labeled primary or secondary antibodies. Such cell sorting
20   methods are standard and are well known to those skilled in the art.

The labeled antibody may be a polyclonal or monoclonal antibody.   In one embodiment, the labeled antibody is a
25   purified labeled antibody. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric
30   antibodies and wholly synthetic antibodies, and fragments thereof.   The detectable marker may be, for example, radioactive or fluorescent. Methods of labeling antibodies are well known in the art.

35   This invention provides a method of inducing differentiation of somatic motor neurons which comprises expressing MNR2 protein in any neural progenitor cells. In an embodiment of the method, expression of MNR2 protein induces expression of

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transcription factors Isl2, Lim 3 and HB9. In a further embodiment of the method of inducing differentiation of somatic motor neurons the neural progenitor cells are spinal cord or hindbrain motor neuron progenitor cells.

This invention provides a transgenic nonhuman mammal which comprises an isolated nucleic acid, including a motor neuron restriction pattern, MNR2 protein, which is a DNA molecule. In an embodiment of the transgenic nonhuman mammal the DNA encoding a MNR2 protein is operatively linked to tissue specific regulatory elements.

This invention provides a method of determining physiological effects of expressing varying levels of MNR2 protein in a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman animals, each nonhuman mammal expressing a different amount of MNR2 protein.

This invention provides a method of producing isolated purified MNR2 protein which comprises: a) inserting a nucleic acid molecule encoding an MNR2 protein into a suitable vector; b) introducing the resulting vector into a suitable host cell; c) selecting the introduced host cell for the expression of the MNR2 protein; d) culturing the selected cell to produce the MNR2 protein; and e) recovering the MNR2 protein produced.

This invention provides a method of inducing differentiation of somatic motor neurons in a subject comprising administering to the subject the purified MNR2 protein in an amount effective to induce differentiation of somatic motor neurons in the subject. In an embodiment, a functionally equivalent analog of MNR2 is administered to the subject. In an embodiment of the method of inducing differentiation of somatic motor neurons in a subject comprising administering to the subject the purified MNR2 protein in an amount effective to induce differentiation of somatic motor

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neurons in the subject, the subject is a mammal. In another
embodiment of the above-described method of inducing
differentiation of somatic motor neurons in a subject, the
subject is a chick, mouse, rat or human.

5

As used herein, "subject" means any animal or artificially
modified animal. Artificially modified animals include, but
are not limited to, SCID mice with human immune systems. In
the preferred embodiment, the subject is a human.

10

This invention provides a pharmaceutical composition
comprising a purified MNR2 protein and a pharmaceutically
acceptable carrier.

15   The invention also provides a pharmaceutical composition
comprising a effective amount of the MNR2 proteins described
above and a pharmaceutically acceptable carrier. In the
subject invention an "effective amount" is any amount of
above-described MNR2 proteins which, when administered to a
20   subject suffering from a disease or abnormality against
which the proteins are determined to be potentially
therapeutic, are effective, causes reduction, remission, or
regression of the disease or abnormality. In the practice
of this invention the "pharmaceutically acceptable carrier"
25   is any physiological carrier known to those of ordinary
skill in the art useful in formulating pharmaceutical
compositions.

In one preferred embodiment the pharmaceutical carrier may
30   be a liquid and the pharmaceutical composition would be in
the form of a solution. In another equally preferred
embodiment, the pharmaceutically acceptable carrier is a
solid and the composition is in the form of a powder or
tablet. In a further embodiment, the pharmaceutical carrier
35   is a gel and the composition is in the form of a suppository
or cream. In a further embodiment the compound may be
formulated as a part of a pharmaceutically acceptable
transdermal patch.

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A solid carrier can include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins.

Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmoregulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be halogenated

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hydrocarbon or other pharmaceutically acceptable propellent.

Liquid pharmaceutical compositions which are sterile
solutions or suspensions can be utilized by for example,
5   intramuscular, intrathecal, epidural, intraperitoneal or
subcutaneous injection. Sterile solutions can also be
administered intravenously. The compounds may be prepared
as a sterile solid composition which may be dissolved or
suspended at the time of administration using sterile water,
10   saline, or other appropriate sterile injectable medium.
Carriers are intended to include necessary and inert
binders, suspending agents, lubricants, flavorants,
sweeteners, preservatives, dyes, and coatings.

15   The above described pharmaceutical composition comprising
MNR2 protein can be administered orally in the form of a
sterile solution or suspension containing other solutes or
suspending agents, for example, enough saline or glucose to
make the solution isotonic, bile salts, acacia, gelatin,
20   sorbitan monoleate, polysorbate 80 (oleate esters of
sorbitol and its anhydrides copolymerized with ethylene
oxide) and the like.

The above described pharmaceutical composition comprising
25   MNR2 protein can also be administered orally either in
liquid or solid composition form. Compositions suitable for
oral administration include solid forms, such as pills,
capsules, granules, tablets, and powders, and liquid forms,
such as solutions, syrups, elixirs, and suspensions. Forms
30   useful for parenteral administration include sterile
solutions, emulsions, and suspensions.

Optimal dosages to be administered may be determined by
those skilled in the art, and will vary with the particular
35   above described pharmaceutical composition comprising MNR2
protein in use, the strength of the preparation, the mode of
administration, and the advancement of the disease condition
or abnormality. Additional factors depending on the

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particular subject being treated will result in a need to
adjust dosages, including subject age, weight, gender, diet,
and time of administration.

5   The subject invention further provides a composition which
comprises an effective amount of a nucleic acid molecule
encoding <u>MNR2</u> capable of being expressed in a suitable host
cell, and a pharmaceutically acceptable carrier.

10   This invention provides a method for treating a subject
afflicted with an abnormality associated with a lack of one
or more normally functioning motor neurons which comprises
introducing an amount of the pharmaceutical composition
comprising of a purified MNR2 protein and a pharmaceutically
15   acceptable carrier effective to generate somatic motor
neurons from undifferentiated motor neuron precursor cells
in the subject, thereby treating the subject afflicted with
the abnormality associated with the lack of one or more
normally functioning motor neurons.

20
       As used herein a "normally functioning motor neuron" is a
motor neuron that can control muscle contraction and respond
to sensory input.

25   As used herein, administering may be effected or performed
using any of the various methods known to those skilled in
the art. The administration may be intravenous,
intraperitoneal,   intrathecal,   intralymphatical,
intramuscular, intralesional, parenteral, epidural,
30   subcutaneous; by infusion, liposome-mediated delivery,
aerosol delivery; topical, oral, nasal, anal, ocular or otic
delivery.

       This invention provides a method of treating a subject
35   afflicted with a neurodegenerative disease which comprises
introducing an amount of the pharmaceutical composition
which comprises a purified MNR2 protein and a
pharmaceutically acceptable carrier effective to generate

somatic motor neurons from undifferentiated precursor motor neuron cells in the subject, thereby treating the subject afflicted with the neurodegenerative disease. In an embodiment of the method of treating a subject with a neurodegenerative disease which comprises introducing an amount of pharmaceutical composition which comprises a purified MNR2 protein and a pharmaceutically acceptable carrier effective to generate somatic motor neurons from undifferentiated precursor motor neuron cells in the subject, the generation of motor neurons from undifferenitated precursor motor neuron cells alleviates a chronic neurodegenerative disease. In an embodiment of the above-described method of treating a subject afflicted with a chronic neurodegenerative disease where the disease is a spinal muscular atrophy. In a further embodiment the of method of treating a subject afflicted with a chronic neurodegenerative disease the disease is amyotrophic lateral sclerosis (Lou Gehrig's Disease).

This invention provides for a method of treating a subject afflicted with an acute nervous system injury which comprises introducing an amount of a pharmaceutical composition which comprises a purified MNR2 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated precursor motor neuron cells in a subject, thereby treating the subject afflicted with the acute nervous system injury.

This invention provides for a method of treating a subject afflicted with an acute nervous system injury which comprises introducing an amount of pharmaceutical composition which comprises a purified MNR2 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated precursor motor neuron cells in a subject, wherein the acute nervous system injury is localized to a specific central axon which comprises surgical implantation of the pharmaceutical compound comprising a MNR2 protein and a pharmaceutically acceptable

carrier effective to generate motor neurons from undifferentiated precursor motor neuron cells located proximal to the specific central axon, so as to alleviate the acute nervous system injury localized to a specific
5   central axon, thereby treating the subject afflicted with the acute nervous system injury.

This invention provides a method for diagnosing a chronic neurodegnerative disease associated with the expression of
10   a MNR2 protein in a sample from a subject which comprises: a. obtaining DNA from the sample of the subject suffering from the chronic neurodegenerative disease; b. performing a restriction digest of the DNA with a panel of restriction enzymes; c. separating the resulting DNA fragments by size
15   fractionation; d. contacting the resulting DNA fragments with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a MNR2 protein, wherein the sequence of a nucleic acid molecule encoding a
20   MNR2 protein is linked at a specific break point to a specified nucleic acid sequence and labeled with a detectable marker; e. detecting labeled bands which have hybridized to the nucleic acid probe capable of specifically hybridizing with a unique sequence included within the
25   sequence of a nucleic acid molecule encoding a MNR2 protein, wherein the sequence of a nucleic acid molecule encoding a MNR2 protein is linked at a specific break point to a specified nucleic acid sequence to create a unique band pattern specific to the DNA of subjects suffering from the
30   chronic neurodegenerative disease; f. preparing DNA obtained from a sample of a subject for diagnosis by steps (a-e); and g. comparing the detected band pattern specific to the DNA obtained from a sample of subjects suffering from the chronic neurodegenerative disease from step (e) and the DNA
35   obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the chronic neurodegenerative disease if the patterns are the

same.

This invention provides a method for diagnosing a chronic neurodegenerative disease associated with the expression of a MNR2 protein in a sample from a subject which comprises: a. obtaining RNA from the sample of the subject suffering from chronic neurodegenerative disease; b. separating the RNA sample by size fractionation; c. contacting the resulting RNA species with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a MNR2 protein, wherein the sequence of a nucleic acid molecule encoding a MNR2 protein is linked at a specific break point to a specified nucleic acid sequence and labeled with a detectable marker; d. detecting labeled bands which have hybridized to the RNA species to create a unique band pattern specific to the RNA of subjects suffering from the chronic neurodegenerative disease; f. preparing RNA obtained from a sample of a subject for diagnosis by steps (a-d); and g. comparing the detected band pattern specific to the RNA obtained from a sample of subjects suffering from the chronic neurodegenerative disease from step (d) and the RNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the chronic neurodegenerative disease if the patterns are the same.

This invention provides a functionally equivalent analog of MNR2 that induces MNR2 differentiation of neural progenitor cells.

This invention provides a functionally equivalent analog of MNR2 that prevents MNR2 differentiation of neural progenitor cells.

This invention provides a method of treating a subject afflicted with a neuromuscular disease which comprises

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introducing an amount of a pharmaceutical composition comprising a purified MNR2 protein and a pharmaceutically acceptable carrier effective to activate acetylcholine to activate muscle cells.

5

In order to facilitate an understanding of the Experimental Details section which follows, certain frequently occurring methods and/or terms are best described in Sambrook, et al.(1989).

10

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the
15 invention as described more fully in the claims which follow thereafter.

This invention provides an isolated nucleic molecule encoding a homeobox, HB9, protein. In an embodiment the isolated nucleic molecule encoding a homeobox HB9, protein
20 is a DNA molecule. In another embodiment the isolated nucleic acid molecule encoding a homeobox, HB9, protein is a cDNA molecule. In a further embodiment the isolated DNA molecule encoding a homeobox, HB9, protein is a RNA
25 molecule. In an embodiment the isolated nucleic acid molecule encoding a motor neuron restricted pattern is operatively linked to a promoter of RNA transcription.

The DNA molecules of the subject invention also include DNA
30 molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified
35 for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides)

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and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide, HB9, and as products for the large scale synthesis of the polypeptide HB9, or fragments thereof, by a variety of recombinant techniques. The DNA molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide HB9 or portions thereof and related products.

In another embodiment the isolated nucleic acid molecule which is a cDNA molecule which, encoding a homebox HB9 protein, encodes a chick HB9 protein. In another embodiment the isolated nucleic acid molecule is a cDNA molecule wherein the nucleic acid molecule encodes a chick HB9 protein comprising the amino acid sequence set forth in SEQ ID NO: 3. In a further embodiment the isolated nucleic acid molecule is a cDNA molecule wherein the nucleic acid molecule encodes a mammalian HB9 protein. In an embodiment the isolated nucleic acid molecule is a cDNA molecule wherein the nucleic acid molecule encodes a mammalian HB9 protein which is a mouse, rat or human protein. In an embodiment the isolated nucleic acid molecule is a cDNA molecule, which comprises the nucleic acid sequence set forth in SEQ ID NO: 4.

This invention provides a vector which comprises the

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isolated nucleic acid encoding a motor neuron restricted pattern, HB9, protein operatively linked to a promoter of RNA transcription. In an embodiment a plasmid comprises the vector which comprises the isolated nucleic acid encoding a

5   homeobox HB9, protein. In another embodiment the plasmid, which comprises the vector which comprises the isolated nucleic acid encoding a chick HB9 pattern, pHB9. (SEQ ID NO.:3)

10   In an embodiment, a full-length cDNA nucleic acid molecule encoding a HB9 protein is inserted into a plasmid and the resulting plasmid is designated as pHB9. The plasmid is with ampicillin resistance and 1.2 kilobase insert is releasable by cleavage with ClaI restriction endonuclease.

15

Numerous vectors for expressing the inventive proteins may be employed. Such vectors, including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses, are well known in the art. For example, one class of vectors

20   utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. Additionally, cells which have stably integrated the DNA into their

25   chromosomes may be selected by introducing one or more markers which allow for the selection of transfected host cells. The markers may provide, for example, prototrophy to an auxotrophic host, biocide resistance or resistance to heavy metals such as copper. The selectable marker gene can

30   be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation.

Regulatory elements required for expression include promoter

35   sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. Additional elements may also be needed for optimal synthesis of mRNA. These additional elements may include splice signals, as

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well as enhancers and termination signals. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general.

These vectors may be introduced into a suitable host cell to form a host vector system for producing the inventive proteins. Methods of making host vector systems are well known to those skilled in the art.

Methods of introducing nucleic acid molecules into cells are well known to those of skill in the art. Such methods include, for example, the use of viral vectors and calcium phosphate co-precipitation.

The "suitable host cell" in which the nucleic acid molecule encoding is HB9 protein capable of being expressed is any cell capable of taking up the nucleic acid molecule and stably expressing the HB9 encoded thereby.

This invention provides a host cell containing the vector which comprises the isolated nucleic acid encoding a homeobox, HB9, protein. In an embodiment the host cell is selected from a group consisting of a bacterial cell, a plant cell, an insect cell and a mammalian cell.

Suitable host cells include, but are not limited to, bacterial cells (including gram positive cells), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to HeLa

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cells, Cos cells, CV1 cells and various primary mammalian
cells.   Numerous mammalian cells may be used as hosts,
including, but not limited to, the mouse fibroblast cell
NIH-3T3 cells, CHO cells, HeLa cells, Ltk⁻ cells and COS

5   cells.   Mammalian cells may be transfected by methods well
known in the art such as calcium phosphate precipitation,
electroporation and microinjection.

This invention provides a method of producing a polypeptide

10   having the biological activity of a mammalian HB9 which
comprises growing host cells selected from a group
consisting of bacterial, plant, insect or mammalian cell,
under suitable conditions permitting production of the
polypeptide.   In another embodiment of the method of

15   producing a polypeptide having the biological activity of a
mammalian HB9 the method further comprises of the recovering
the produced polypeptide.

This invention provides an isolated nucleic acid molecule of

20   at least 15 contiguous nucleotides capable of specifically
hybridizing with a unique sequence included within the
sequence of the nucleic acid molecule encoding a homeobox,
HB9, protein.   In an embodiment the isolated nucleic acid
molecule of at least 15 contiguous nucleotides capable of

25   specifically hybridizing with a unique sequence included
within the sequence of the nucleic acid molecule encoding a
homeobox, HB9, protein is a DNA molecule.   In another
embodiment the isolated nucleic acid molecule of at least 15
contiguous nucleotides capable of specifically hybridizing

30   with a unique sequence included within the sequence of the
nucleic acid molecule encoding a homebox, HB9, protein, is
a RNA molecule.

This invention provides an isolated nucleic acid molecule

35   capable of specifically hybridizing with a unique sequence
included within the sequence of a nucleic acid molecule
which is complementary to the nucleic acid molecule encoding
a homebox, HB9, protein.   In an embodiment the isolated

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nucleic acid molecule capable of specifically hybridizing
with a unique sequence included within the sequence of a
nucleic acid molecule which is complementary to the nucleic
acid molecule encoding a homeobox, HB9, protein, is a DNA
5   molecule.   In another embodiment the isolated nucleic acid
molecule capable of specifically hybridizing with a unique
sequence included within the sequence of a nucleic acid
molecule which is complementary to the nucleic acid molecule
encoding a homeobox, HB9, protein is a RNA molecule.

10

One of ordinary skill in the art will easily obtain unique
sequences from the cDNA cloned in the plasmid. Such unique
sequences may be used as probes to screen various mammalian
cDNA libraries and genomic DNAs, e.g. mouse, rat and bovine,
15   to obtain homologous nucleic acid sequences and to screen
different cDNA tissue libraries to obtain isoforms of the
obtained nucleic acid sequences. Nucleic acid probes from
the cDNA cloned in the pHB9 plasmid may further be used to
screen other human tissue cDNA libraries to obtain isoforms
20   of the nucleic acid sequences encoding HB9 protein as well
as to screen human genomic DNA to obtain the analogous
nucleic acid sequences. The homologous nucleic acid
sequences and isoforms may be used to produce the proteins
encoded thereby.

25

As used herein, "capable of specifically hybridizing" means
capable of binding to an mRNA molecule encoding a HB9 but
not capable of binding to an mRNA molecule encoding a HB9
receptor protein.

30

This invention provides an antisense oligonucleotide having
a nucleic acid sequence capable of specifically hybridizing
to an mRNA molecule encoding a HB9 protein.   In an
embodiment the antisense oligonucleotide has a nucleic acid
35   sequence capable of specifically hybridizing to the isolated
cDNA molecule encoding a homeobox, HB9, protein. In an
embodiment the antisense oligonucleotide has a nucleic acid
sequence capable of specifically hybridizing to an isolated

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RNA molecule encoding a homeobox, HB9 protein.

This invention provides a purified HB9 protein. In an
embodiment the purified HB9 protein is encoded by an
isolated nucleic acid encoding a homeobox, HB9, protein. In
another embodiment the HB9 protein unique polypeptide
fragment of the purified HB9 protein. In an embodiment the
purified HB9 protein has substantially the same amino acid
sequence as set forth in SEQ ID NO: 3. In a further
embodiment the purified HB9 protein having a nucleic acid
sequence as set forth in SEQ ID NO: 4. In another
embodiment the purified HB9 protein has an amino acid
sequence as set forth in SEQ ID NO: 3. In a further
embodiment, the MNR2 protein is a vertebrate HB9 protein.
In an embodiment the purified vertebrate HB9 protein having
an amino acid sequence as set forth in SEQ ID NO: 3 is a
chick, mouse or rat HB9 protein.

As used herein, an HB9 protein having "substantially the
same" amino acid sequences as set forth in SEQ ID NO: 3 is
encoded by a nucleic acid encoding HB9, said nucleic acid
having 100% identity in the homeodomain regions, that is
those regions coding the protein, and said nucleic acid may
vary in the nucleotides in the non-coding regions.

This invention provides a monoclonal antibody directed to an
epitope of an HB9 protein. In an embodiment the monoclonal
antibody is directed to a chick, mouse or rat HB9 protein.

This invention provides a polyclonal antibody directed to an
epitope of the purified HB9 protein having the amino
sequence as set forth in SEQ ID No: 3. In a further
embodiment the monoclonal or polyclonal antibodies are
directed to the HB9 protein, having the amino acid sequence
as set forth in SEQ ID NO: 3.

Polyclonal antibodies may be produced by injecting a host
animal such as rabbit, rat, goat, mouse or other animal with

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the immunogen of this invention, e.g. a purified mammalian HB9 protein or a purified human HB9 protein. The sera are extracted from the host animal and are screened to obtain polyclonal antibodies which are specific to the immunogen. Methods of screening for polyclonal antibodies are well known to those of ordinary skill in the art such as those disclosed in Harlow & Lane, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY: 1988) the contents of which are hereby incorporated by reference.

The monoclonal antibodies may be produced by immunizing for example, mice with an immunogen. The mice are inoculated intraperitoneally with an immunogenic amount of the above-described immunogen and then boosted with similar amounts of the immunogen. Spleens are collected from the immunized mice a few days after the final boost and a cell suspension is prepared from the spleens for use in the fusion.

Hybridomas may be prepared from the splenocytes and a murine tumor partner using the general somatic cell hybridization technique of Kohler, B. and Milstein, C., Nature (1975) 256: 495-497. Available murine myeloma lines, such as those from the American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD 20852 USA, may be used in the hybridization. Basically, the technique involves fusing the tumor cells and splenocytes using a fusogen such as polyethylene glycol. After the fusion the cells are separated from the fusion medium and grown in a selective growth medium, such as HAT medium, to eliminate unhybridized parent cells. The hybridomas may be expanded, if desired, and supernatants may be assayed by conventional immunoassay procedures, for example radioimmunoassay, using the immunizing agent as antigen. Positive clones may be characterized further to determine whether they meet the criteria of the invention antibodies.

Hybridomas that produce such antibodies may be grown in

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In the practice of the subject invention any of the above-described antibodies may be labeled with a detectable marker. In one embodiment, the labeled antibody is a purified labeled antibody. As used in the subject invention, the term "antibody" includes, but is not limited to, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and binding fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof.

Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. A "detectable moiety" which functions as detectable labels are well known to those of ordinary skill in the art and include, but are not limited to, a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase, ß-galactosidase, fluorescein or steptavidin/biotin. Methods of labeling antibodies are well known in the art.

Determining whether the antibody forms such a complex may be accomplished according to methods well known to those skilled in the art. In the preferred embodiment, the determining is accomplished according to flow cytometry methods.

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The antibody may be bound to an insoluble matrix such as that used in affinity chromatography. As used in the subject invention, isolating the cells which form a complex with the immobilized monoclonal antibody may be achieved by

5   standard methods well known to those skilled in the art. For example, isolating may comprise affinity chromatography using immobilized antibody.

Alternatively, the antibody may be a free antibody. In this

10   case, isolating may comprise cell sorting using free, labeled primary or secondary antibodies. Such cell sorting methods are standard and are well known to those skilled in the art.

15   The labeled antibody may be a polyclonal or monoclonal antibody. In one embodiment, the labeled antibody is a purified labeled antibody. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody"

20   includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. The detectable marker may be, for example, radioactive or fluorescent. Methods of labeling antibodies

25   are well known in the art.

This invention provides a method of inducing differentiation of somatic motor neurons which comprises expressing MNR2 protein in any neural progenitor cells. In an embodiment of

30   the method, expression of HB9 protein induces expression of transcription factors Isl2, Lim 3 and HB9. In a further embodiment of the method of inducing differentiation of somatic motor neurons the neural progenitor cells are spinal cord or hindbrain motor neuron progenitor cells.

35

This invention provides a transgenic nonhuman mammal which comprises an isolated nucleic acid, including a motor neuron restriction pattern, HB9 protein, which is a DNA molecule.

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In an embodiment of the transgenic nonhuman mammal the DNA encoding a HB9 protein is operatively linked to tissue specific regulatory elements.

5   This invention provides a method of determining physiological effects of expressing varying levels of HB9 protein in a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman animals, each nonhuman mammal expressing a different amount of HB9

10   protein.

This invention provides a method of producing isolated purified HB9 protein which comprises: a) inserting a nucleic acid molecule encoding an HB9 protein into a suitable vector; b) introducing the resulting vector into a suitable

15   host cell; c) selecting the introduced host cell for the expression of the HB9 protein; d) culturing the selected cell to produce the HB9 protein; and e) recovering the HB9 protein produced.

20

This invention provides a method of inducing differentiation of somatic motor neurons in a subject comprising administering to the subject the purified HB9 protein in an amount effective to induce differentiation of somatic motor

25   neurons in the subject. In an embodiment, a functionally equivalent analog of HB9 is administered to the subject. In an embodiment of the method of inducing differentiation of somatic motor neurons in a subject comprising administering to the subject the purified HB9 protein in an amount

30   effective to induce differentiation of somatic motor neurons in the subject, the subject is a mammal. In another embodiment of the above-described method of inducing differentiation of somatic motor neurons in a subject, the subject is a chick, mouse, rat or human.

35

As used herein, "subject" means any animal or artificially modified animal. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. In

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the preferred embodiment, the subject is a human.

This invention provides a pharmaceutical composition comprising a purified HB9 protein and a pharmaceutically acceptable carrier.

The invention also provides a pharmaceutical composition comprising a effective amount of the HB9 proteins described above and a pharmaceutically acceptable carrier. In the subject invention an "effective amount" is any amount of above-described HB9 proteins which, when administered to a subject suffering from a disease or abnormality against which the proteins are determined to be potentially therapeutic, are effective, causes reduction, remission, or regression of the disease or abnormality. In the practice of this invention the "pharmaceutically acceptable carrier" is any physiological carrier known to those of ordinary skill in the art useful in formulating pharmaceutical compositions.

In one preferred embodiment the pharmaceutical carrier may be a liquid and the pharmaceutical composition would be in the form of a solution. In another equally preferred embodiment, the pharmaceutically acceptable carrier is a solid and the composition is in the form of a powder or tablet. In a further embodiment, the pharmaceutical carrier is a gel and the composition is in the form of a suppository or cream. In a further embodiment the compound may be formulated as a part of a pharmaceutically acceptable transdermal patch.

A solid carrier can include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is

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mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size desired.   The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid
5   carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins.

10   Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions.   The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both or
15   pharmaceutically acceptable oils or fats.   The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-
20   regulators.   Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric
25   alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil).   For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form
30   compositions for parenteral administration.   The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellent.

Liquid pharmaceutical compositions which are sterile
35   solutions or suspensions can be utilized by for example, intramuscular, intrathecal, epidural, intraperitoneal or subcutaneous injection.   Sterile solutions can also be administered intravenously.   The compounds may be prepared

as a sterile solid composition which may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium. Carriers are intended to include necessary and inert

5   binders, suspending agents, lubricants, flavorants, sweeteners, preservatives, dyes, and coatings.

The above described pharmaceutical composition comprising HB9 protein can be administered orally in the form of a

10   sterile solution or suspension containing other solutes or suspending agents, for example, enough saline or glucose to make the solution isotonic, bile salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene

15   oxide) and the like.

The above described pharmaceutical composition comprising HB9 protein can also be administered orally either in liquid or solid composition form. Compositions suitable for oral

20   administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

25

Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular above described pharmaceutical composition comprising HB9 protein in use, the strength of the preparation, the mode of

30   administration, and the advancement of the disease condition or abnormality. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

35

The subject invention further provides a composition which comprises an effective amount of a nucleic acid molecule encoding HB9 capable of being expressed in a suitable host

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cell, and a pharmaceutically acceptable carrier.

This invention provides a method for treating a subject afflicted with an abnormality associated with a lack of one or more normally functioning motor neurons which comprises introducing an amount of the pharmaceutical composition comprising of a purified HB9 protein and a pharmaceutically acceptable carrier effective to generate somatic motor neurons from undifferentiated motor neuron precursor cells in the subject, thereby treating the subject afflicted with the abnormality associated with the lack of one or more normally functioning motor neurons.

As used herein a "normally functioning motor neuron" is a motor neuron that can control muscle contraction and respond to sensory input.

As used herein, administering may be effected or performed using any of the various methods known to those skilled in the art. The administration may be intravenous, intraperitoneal, intrathecal, intralymphatical, intramuscular, intralesional, parenteral, epidural, subcutaneous; by infusion, liposome-mediated delivery, aerosol delivery; topical, oral, nasal, anal, ocular or otic delivery.

This invention provides a method of treating a subject afflicted with a neurodegenerative disease which comprises introducing an amount of the pharmaceutical composition which comprises a purified HB9 protein and a pharmaceutically acceptable carrier effective to generate somatic motor neurons from undifferentiated precursor motor neuron cells in the subject, thereby treating the subject afflicted with the neurodegenerative disease. In an embodiment of the method of treating a subject with a neurodegenerative disease which comprises introducing an amount of pharmaceutical composition which comprises a purified HB9 protein and a pharmaceutically acceptable

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carrier effective to generate somatic motor neurons from undifferentiated precursor motor neuron cells in the subject, the generation of motor neurons from undifferenitated precursor motor neuron cells alleviates a
5   chronic neurodegenerative disease. In an embodiment of the above-described method of treating a subject afflicted with a chronic neurodegenerative disease where the disease is a spinal muscular atrophy. In a further embodiment the of method of treating a subject afflicted with a chronic
10   neurodegenerative disease the disease is amyotrophic lateral sclerosis (Lou Gehrig's Disease).

This invention provides for a method of treating a subject afflicted with an acute nervous system injury which
15   comprises introducing an amount of a pharmaceutical composition which comprises a purified HB9 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated precursor motor neuron cells in a subject, thereby treating the subject afflicted
20   with the acute nervous system injury.

This invention provides for a method of treating a subject afflicted with an acute nervous system injury which comprises introducing an amount of pharmaceutical
25   composition which comprises a purified HB9 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated precursor motor neuron cells in a subject, wherein the acute nervous system injury is localized to a specific central axon which comprises
30   surgical implantation of the pharmaceutical compound comprising a HB9 protein and a pharmaceutically acceptable carrier effective to generate motor neurons from undifferentiated precursor motor neuron cells located proximal to the specific central axon, so as to alleviate
35   the acute nervous system injury localized to a specific central axon, thereby treating the subject afflicted with the acute nervous system injury.

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This invention provides a method for diagnosing a chronic neurodegnerative disease associated with the expression of a HB9 protein in a sample from a subject which comprises: a. obtaining DNA from the sample of the subject suffering from the chronic neurodegenerative disease; b. performing a restriction digest of the DNA with a panel of restriction enzymes; c. separating the resulting DNA fragments by size fractionation; d. contacting the resulting DNA fragments with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a HB9 protein, wherein the sequence of a nucleic acid molecule encoding a HB9 protein is linked at a specific break point to a specified nucleic acid sequence and labeled with a detectable marker; e. detecting labeled bands which have hybridized to the nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a HB9 protein, wherein the sequence of a nucleic acid molecule encoding a HB9 protein is linked at a specific break point to a specified nucleic acid sequence to create a unique band pattern specific to the DNA of subjects suffering from the chronic neurodegenerative disease; f. preparing DNA obtained from a sample of a subject for diagnosis by steps (a-e); and g. comparing the detected band pattern specific to the DNA obtained from a sample of subjects suffering from the chronic neurodegenerative disease from step (e) and the DNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the chronic neurodegenerative disease if the patterns are the same.

This invention provides a method for diagnosing a chronic neurodegenerative disease associated with the expression of a HB9 protein in a sample from a subject which comprises: a. obtaining RNA from the sample of the subject suffering from chronic neurodegenerative disease; b. separating the RNA

sample by size fractionation; c. contacting the resulting RNA species with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a HB9 protein, wherein the sequence of a nucleic acid molecule encoding a HB9 protein is linked at a specific break point to a specified nucleic acid sequence and labeled with a detectable marker; d. detecting labeled bands which have hybridized to the RNA species to create a unique band pattern specific to the RNA of subjects suffering from the chronic neurodegenerative disease; f. preparing RNA obtained from a sample of a subject for diagnosis by steps (a-d); and g. comparing the detected band pattern specific to the RNA obtained from a sample of subjects suffering from the chronic neurodegenerative disease from step (d) and the RNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the chronic neurodegenerative disease if the patterns are the same.

This invention provides a functionally equivalent analog of HB9 that induces HB9 differentiation of neural progenitor cells.

This invention provides a functionally equivalent analog of HB9 that prevents HB9 differentiation of neural progenitor cells.

This invention provides a method of treating a subject afflicted with a neuromuscular disease which comprises introducing an amount of a pharmaceutical composition comprising a purified HB9 protein and a pharmaceutically acceptable carrier effective to activate acetylcholine to activate muscle cells.

This invention provides a method of treating an embryo afflicted with sacral agenesis which comprises introducing

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an isolated nucleic molecule encoding a homeobox, HB9, protein.

This invention provides a method of treating an embryo afflicted with sacral agenesis which comprises introducing an amount of a pharmaceutical composition comprising a purified HB9 protein and a pharmaceutically acceptable carrier effective to activate acetylcholine to activate muscle cells.

A method of treating an embryo lacking HB9 expression which comprises introducing an isolated nucleic molecule encoding a homeobox, HB9, protein.

A method of treating an embryo lacking HB9 expression which comprises introducing an amount of a pharmaceutical composition comprising a purified HB9 protein and a pharmaceutically acceptable carrier effective to activate acetylcholine to activate muscle cells.

In order to facilitate an understanding of the Experimental Details section which follows, certain frequently occurring methods and/or terms are best described in Sambrook, et al.(1989).

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

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EXPERIMENTAL DETAILS

FIRST SET OF EXPERIMENTS

5   **Single-Cell Library Screen**
       Culture of stage 10 chick (Hamburger and Hamilton, 1951) [i]
       explants was performed as described (Yamada et al.,1993).
       After 24 hr, explants were enzymatically dissociated and
       single cells isolated. The synthesis and amplification of
10   cDNAs isolated from single cells was performed essentially
       as in Dulac and Axel (1995). Single cell-derived cDNAs were
       analyzed using Isl1, Isl2, HB9 and Lim2 probes. The
       efficiency of cDNA amplification was assessed using S17. A
       single cell cDNA sample that revealed Isl1, Isl2 and HB9
15   hybridization was used to prepare a cDNA library in λZapII
       (Stratagene). Approximately 2000 plaques were screened with
       cDNAs from a motor neuron and Lim2^{+} neuron. Plaques
       hybridizing selectively with the motor neuron probe were
       isolated, cloned and analyzed by in situ hybridization.
20   This screen generated 2 clones that encoded MNR2.

       As used herein, "[i] explants" are intermediate neural plate
       explants.

25   **Isolation of MNR2 and HB9**
       A single motor neuron-derived MNR2 cDNA clone was used to
       isolate a longer MNR2 cDNA. A genomic MNR2 clone containing
       the 5' coding region of MNR2 was ligated to the MNR2 cDNA to
       generate full-length chick MNR2. A chick HB9 cDNA was
30   isolated from a spinal cord cDNA library using a PCR probe
       derived from the HB9 homeobox. A full length chick HB9 clone
       was constructed by ligating genomic HB9 coding sequence to
       the HB9 cDNA. Genbank accession numbers; chick MNR2
       AF066860, chick HB9 AF066861.

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Recombinant Retroviral Vectors
As used herein, a recombinant nucleic acid molecule is a
nucleic acid molecule which does not occur as an individual
molecule in nature and which is obtained through the use of
5   recombinant technology.

Retroviral vectors were generated by subcloning chick cDNAs
into either RCASBP(A) or RCASBP(B) vectors (Hughes et al.,
1987; Morgan and Fekete, 1996).   The RCAS(B) MNR2 3'Δ
10   construct contained the coding region of MNR2 but lacked the
3' non-coding region.   Viral supernatants (Morgan and
Fekete, 1996) were applied to stage 5-6 chick embryos in
ovo.   In double infections, viral stocks derived from
vectors encoding A or B envelope glycoproteins were mixed
15   prior to in ovo application.   Embryos were analyzed at
stages 17-27.

Immunocytochemistry and In Situ Hybridization Histochemistry
Rabbit antisera were generated against a GST-chick MNR2 NH_2
20   fusion protein and against a KLH-conjugated chick HB9 NH_2
peptide (MEKSKNFRIDALLA).   Mouse antisera and monoclonal
antibodies were also generated against MNR2 protein and HB9
protein.   Rabbit anti-Brn3.0 (Fedtsova and Turner, 1997);
guinea pig anti Isl1/2 (Morton and T.M. Jessell unpublished
25   data).   For other antibody reagents see Ericson et al.,
(1997) and Liem et al., (1997).   Immunohistochemistry was
performed as described (Yamada et al., 1993). Double and
triple-label analyses were performed with a Bio-Rad 1024
Confocal Microscope using Cy3, Cy5 and FITC-conjugated
30   secondary antibodies (Jackson, Inc.).   In situ hybridization
was performed as described (Schaeren-Wiemers and Gerfin-
Moser, 1993; Tsuchida et al., 1994) using MNR2 and ChAT
(Yamada, et al., 1993) probes.   The 3' non-coding MNR2 probe
was the 300 bp SmaI-EcoRI fragment.
35

BrdU Incorporation into Neural Neurons
BrdU incorporation was analyzed after application of 100uM
BrdU to stage 18 embryos in ovo and subsequent incubation at

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37°C for 30 or 45 minutes.

Retrograde Labeling of Spinal Cord Cells

RCAS(B)MNR2-infected stage 24-26 embryos were dissected,
5   ventral roots exposed and labeled by application of FITC-
Dextran (Molecular Probes) (Varela-Echavarria et al., 1996).
Embryos were incubated for 1 hour before analysis.

10   **MATERIALS AND METHODS**

Isolation of MNR2

To identify genes expressed by somatic motor neuron
progenitors, we performed a PCR-based differential screen
15   (Dulac and Axel, 1995) of individual newly-differentiated
motor neurons on the premise that genes expressed by motor
neuron progenitors might persist transiently in post-mitotic
motor neurons. Chick neural plate ([i]) explants (Yamada et
al, 1993) were grown in vitro for 24h, alone or with a
20   concentration of Shh that induces somatic motor neurons
(Ericson et al. 1997). cDNAs isolated from single cells
derived from [i] explants exposed to Shh were hybridized
with probes for three homeobox genes Isl1, Isl2 and HB9,
that define post-mitotic somatic motor neurons (Fig 1A).
25   Single cell cDNA samples derived from [i] explants grown
alone were hybridized with Lim2, a homeobox gene marker of
spinal interneurons (Fig 1A). A cDNA population amplified
from a single Isl1$^+$, Isl2$^+$, HB9$^+$ motor neuron (Fig 1A) was
used to prepare a cDNA library that was screened with probes
30   derived from a single in vitro generated motor neuron or
Lim2$^+$ neuron. cDNAs isolated from plaques that hybridized
selectively with the motor neuron-derived probe were cloned
and sequenced.   As used herein, "[i] explants" are
intermediate neural plate explants.
35

Several genes identified in this manner were expressed in a
motor neuron restricted pattern and we describe here the
function of one of these, MNR2. MNR2 is a homeobox gene

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that encodes a homeodomain almost identical to that encoded by <u>HB9</u> (Fig 1B; Harrison et al., 1994), a gene whose neural expression is restricted to motor neurons (see below, Pfaff et al. 1996, Saha et al., 1997). The MNR2 sequence diverges

5   from HB9 outside the homeodomain (Fig 1B; data not shown).

MNR2 is Expressed by Somatic Motor Neuron Progenitors

The pattern of neural expression of <u>MNR2</u> was examined in chick embryos from stages 10 to 25. <u>MNR2</u> expression was

10   first detected at stage 12 in cells near the floor plate (Fig 1C). At this stage, no motor neurons have been generated (Langman et al., 1966) and Isl1 is not yet expressed (Ericson et al., 1992). The onset of <u>MNR2</u> expression coincides with the time that motor neuron

15   progenitors acquire independence from Shh signaling (Ericson et al., 1996). Between stages 15-20, <u>MNR2</u> expression was restricted to cells in the ventral spinal cord and caudal hindbrain, some of which were located medially and others laterally in a domain that overlaps with post-mitotic <u>Isl1</u>$^+$

20   motor neurons (Fig 1 D, 1E; data not shown).

We next examined whether, as predicted by the design of the differential screen, MNR2 is induced by Shh. To test this, [i] explants were exposed to Shh and the expression of MNR2

25   and Isl1/2 determined. Induction of MNR2 was detected 16h after exposure to Shh (data not shown) and by 24h, 70 $\pm$ 12 (mean $\pm$ SEM; n=4) MNR2$^+$ cells were detected (Fig 1 F, 1G). In addition, 32% of the induced MNR2$^+$ cells did not express Isl1/2 (Fig 1 G, 1H), consistent with the earlier onset of

30   <u>MNR2</u> expression in vivo. These results show that Shh induces MNR2.

To provide direct evidence that MNR2 is expressed by mitotic cells, we exposed stage 18 embryos to BrdU for 30 min and

35   monitored the expression of MNR2 and Isl1 by BrdU$^+$ cells. Many MNR2$^+$, BrdU$^+$ cells (Fig 2A) but no Isl1 , BrdU cells (Fig 2B) were detected. In addition, a subset of MNR2$^+$ progenitors coexpressed the M-phase marker MPM2 (Fig 2C;

Westendorf et al., 1994). MNR2^{+} progenitors coexpressed Pax6
(Fig 2D; data not shown) but were located dorsal to the
Nkx2.2[+] progenitor domain (Fig 2E), supporting the idea that
MNR2 expression is restricted to the progenitors of somatic
5   motor neurons.   MNR2 was also detected in post-mitotic
somatic motor neurons in the spinal cord and hindbrain but
not in visceral motor neurons (Fig 1E , Fig 2 F and data not
shown).   Thus, MNR2 expression is restricted to domains of
somatic motor neuron generation.
10

The temporal profile of MNR2 expression was compared with
that of other homeodomain proteins that define somatic motor
neurons.   At spinal cord levels, the initial migration of
newly-generated motor neurons occurs in a mediolateral plane
15   (Leber and Sanes, 1995) and thus the more lateral the
position of a motor neuron, the more advanced is its state
of differentiation.   Between stages 15 and 20, MNR2 was
detected medially in progenitor cells as well as laterally
in post-mitotic motor neurons (Fig 2 G-K, data not shown).
20   The expression of Isl1 was restricted to post-mitotic motor
neurons (Fig 2 G, 2K; Ericson et al., 1992) but appeared
prior to HB9 and Isl2 (Fig 2H, 2I, 2K; data not shown). In
these laterally positioned motor neurons MNR2 expression was
markedly decreased or absent (Fig 2 H, 2I, 2K) indicating
25   that MNR2 expression is transient. Lim3 is expressed by
post-mitotic somatic motor neurons (Figure 2 J, 2K; Tsuchida
et al., 1994) but is also detected in Pax6^{+} ventral
progenitors and in these cells appears soon after MNR2 (Fig
2 J, 2K; data not shown).   Thus, somatic motor neuron
30   progenitors coexpress Pax6, MNR2 and Lim3.

**MNR2 Induces the Expression of Somatic Motor Neuron
Transcription Factors**
If MNR2 has a critical role in the Shh-induced pathway of
35   motor neuron generation, we reasoned that its misexpression
might be sufficient to promote motor neuron differentiation
in neural cells that have not been exposed to adequate
levels of Shh and perhaps even in dorsal progenitor cells

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that are exposed to antagonistic BMP signals. To test this idea we misexpressed MNR2 in the neural tube using a replication competent retroviral vector (Morgan and Fekete, 1996). Embryos were infected with MNR2 virus at stages 5-6,
5   permitted to develop until stages 20-27 and the resulting pattern of motor neuron differentiation monitored by expression of Isl1, Isl2, HB9, and Lim3. The expression of Isl2 and HB9 is restricted to somatic motor neurons (Tsuchida et al. 1994; Pfaff et al. 1996; data not shown)
10   whereas Lim3 is also expressed by ventral (V2) interneurons that are generated immediately dorsal to motor neurons (Fig 2 J, 2K; Ericson et al. 1997). Isl1 is also expressed by dorsal (D2) interneurons (Fig 2 G, 2K; Liem et al. 1997).

15   In MNR2-infected embryos examined at stage 23, ectopic MNR2$^+$ cells were detected in a mosaic pattern throughout the spinal cord (Fig 3A; data not shown). In dorsal regions that contained a high density of MNR2$^+$ cells, many ectopic Isl1$^+$, Isl2$^+$, HB9$^+$ and Lim3 $^+$ cells were detected (Fig 3 B-E). All
20   ectopic Isl1$^+$, Lim3$^+$, Isl2 $^+$ and HB9 $^+$ cells coexpressed MNR2 (Fig 3F; data not shown) indicating that MNR2 acts cell-autonomously to activate the expression of these homeodomain proteins. To control for the specificity of MNR2 activity, we misexpressed Lmx1b and Hoxc6, two other homeobox genes
25   present in the spinal cord (Burke and Tabin 1996; Riddle et al. 1995). Neither Lmx1b nor Hoxc6 induced MNR2, Lim3, Isl1, Isl2 or HB9 (data not shown). Thus, the activity of MNR2 is not mimicked by divergent homeodomain proteins.

30   We next examined whether the ability of MNR2 to induce motor neuron markers in the dorsal spinal cord is associated with a change in the early dorsal character of neural progenitor cells. The ectopic dorsal expression of MNR2 did not repress Pax7 (Fig 3G), a marker of dorsal progenitor cells (Ericson
35   et al., 1996). The detection of Isl2$^+$, HB9$^+$ and Lim3$^+$ cells in the extreme dorsal region of the spinal cord (Fig 3 C-E) also provides evidence that MNR2 can impose a somatic motor neuron character on neural cells that have been exposed to

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BMP signals (Liem et al., 1997). Consistent with this, there was no dorsoventral restriction in the positions at which cells expressing these ectopic homeodomain protein markers were found (Fig 3 B-E; data not shown). Ectopic
5   expression of Lim3 and HB9 was also detected in dorsal root ganglion neurons (data not shown), indicating that MNR2 can also activate somatic motor neuron markers in peripheral neurons.

10   The ability of MNR2 to induce motor neuron transcription factors in the dorsal spinal cord leaves open the issue of whether ventral progenitor cells respond to MNR2 with the expression of motor neuron markers. The detection of a high incidence of MNR2-induced Isl1$^+$, Isl2$^+$ and HB9$^+$ cells below
15   the Pax7 boundary (Fig. 3B-D, 3G) suggested that this is the case. Nevertheless, it remained possible that the ectopic location of these neurons arose through their dorsal migration from the normal domain of somatic motor neuron generation. We therefore examined whether MNR2 can induce
20   motor neuron transcription factors in ventral progenitor cells generated in neural plate explants. Embryos were infected with MNR2 virus at stages 4-5 and permitted to develop until stage 10, at which time [i] explants were isolated and grown in vitro for 24h with 0.5nM Shh. This
25   concentration of Shh is sufficient to repress Pax7 and generate a ventral progenitor cell state (Ericson et al., 1996), but is below the threshold for motor neuron induction (Ericson et al., 1997). [i] explants isolated from MNR2-infected embryos contained many MNR2$^+$ cells and ~25% of these
30   expressed Isl1/2 (Fig 3 H, 3I). In contrast, [i] explants derived from uninfected embryos and exposed to 0.5 nM Shh did not generate MNR2$^+$ cells (Fig 3J) or Isl1/2 neurons (data not shown; Ericson et al., 1997). These results indicate that MNR2 can also direct motor neuron
35   differentiation in ventral progenitor cells.

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MNR2 Acts in the Context of a General Program of Neurogenesis

The expression of motor neuron transcription factors was detected in only a minority of ectopic MNR2$^+$ cells (Fig 3 A-3F). In considering what might account for this restriction we noted that the ectopic expression of Isl1, Isl2 and HB9 coincided with the location of post-mitotic neurons (Fig 3 B-E). This observation, taken together with the normal restriction of Isl1, Isl2 and HB9 to post-mitotic motor neurons (Fig 2K) raised the possibility that the induction of these homeodomain proteins by MNR2 might require cell cycle exit and the acquisition of a generic neuronal character.

To examine this possibility we first determined the proportion of ectopic MNR2$^+$ cells that expressed Cyn1, a marker expressed by all spinal neurons soon after cell cycle exit (Ericson et al., 1997). At stages 20-23, only ~10% of ectopic MNR2$^+$ cells expressed Cyn1 (Fig 4 A, 4B), indicating that most ectopic MNR2$^+$ are progenitors. In contrast, >98% of MNR2-induced ectopic Isl1$^+$, Isl2 and $^+$HB9 cells coexpressed Cyn1 (Fig 4 C-4E; data not shown). These findings suggest that the ectopic induction of Isl1, Isl2 and HB9 by MNR2 requires the acquisition of a post-mitotic neuronal character, thus conforming to the normal profile of expression of these three proteins in somatic motor neurons. Lim3, in contrast, is normally expressed by ventral progenitors as well as by post-mitotic motor neurons. Many MNR2-induced Lim3$^+$ cells could be labeled after a brief BrdU pulse (Fig 4 F), ~70% of MNR2-induced ectopic Lim3$^+$ cells coexpressed Cyn1 (data not shown) and only 55% of MNR2-induced Lim3$^+$ cells coexpressed Isl1 (Fig 4 E, 4G). Thus MNR2 induces ectopic Lim3 expression in neural progenitors and post-mitotic neurons. These findings show that the normal temporal relationship between cell cycle exit and transcription factor expression is preserved in ectopic MNR2-induced neurons. In a neuronal context then, MNR2 is an efficient inducer of somatic motor neuron transcription

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factors: 91% of MNR2$^+$ neurons expressed Isl1, 79% expressed Lim3 and ~40% expressed Isl2 and HB9 (Fig 4H).

MNR2 Expression is Autoregulated

The onset of MNR2 expression occurs at the time that motor neuron progenitors attain independence from Shh signaling (Ericson et al., 1996). This finding raises the possibility that the progression of motor neuron progenitors to a Shh-independent state involves the expression of MNR2. One mechanism by which MNR2 might establish Shh-independence is through the activation of its own expression.

To test this, we infected embryos with a MNR2 3'Δ construct lacking 3' non-coding sequence and monitored the expression of endogenous MNR2 using a 3' non-coding probe. Retrovirally-introduced MNR2 3'Δ induced endogenous MNR2 in dorsal spinal cord cells (Fig 4 I, 4J). Moreover, the ectopic expression of MNR2 was not restricted to neural cells (Fig 4J), in contrast to the neural restriction in the MNR2-induced ectopic expression of Lim3, Isl1, Isl2 and HB9 (Fig 3; data not shown). These results support the idea that MNR2 expression and autoactivation underlies the progression of motor neuron progenitors to a Shh-independent state.

MNR2 Induces Later Features of Motor Neuron Differentiation

The sufficiency of MNR2 as an inducer of somatic motor neuron transcription factors prompted us to examine whether these neurons exhibit other aspects of the motor neuron phenotype. We first examined if MNR2 can induce the expression of ChAT, the gene encoding the rate limiting enzyme in the synthesis of acetylcholine, the motor neuron neurotransmitter. In control embryos, ChAT expression was restricted to the region occupied by motor neuron cell bodies and their dendrites (Fig 5A, 5C, 5E). In contrast, in MNR2-infected embryos, ChAT was expressed ectopically by cells in the dorsal spinal cord (Fig 5B, 5D, 5E). Thus,

MNR2 induces the gene that defines the transmitter phenotype
of motor neurons.

5   We next examined whether neurons in the dorsal spinal cord
       that express MNR2 acquire the motor neuron-specific feature
       of an axonal projection into the ventral root. To assess
       this we labeled spinal neurons retrogradely by application
       of FITC-Dextran to the ventral roots of stage 25 embryos.
       In control embryos, labeled neurons were restricted to the
10   ventral spinal cord (Fig 5 F, 5J).   In MNR2-infected
       embryos, many ectopic dorsal MNR2$^+$, Isl1/2$^+$ neurons were
       labeled with FITC-Dextran and these neurons projected axons
       towards the ventral root exit point (Fig 5 G-J).   In
       addition, ectopic expression of SC1, a surface marker of
15   motor neurons (Tanaka and Obata, 1984) was detected in MNR2-
       infected but not in control embryos (data not shown). These
       findings show that MNR2 induces later phenotypic features of
       somatic motor neurons.

20   **Ectopic Expression of MNR2 Represses Interneuron Fates**
       The detection of ectopic motor neuron differentiation in
       dorsally-located neurons raised the question of whether the
       interneuronal character of spinal cord neurons is suppressed
       by MNR2. To address this issue we analyzed the expression
25   of homeodomain proteins that define four classes of
       interneurons generated in domains dorsal to motor neurons.
       Dorsally, D1 neurons express LH2 (Liem et al., 1997) and
       many D2 neurons express Brn3.0 (Fedtsova and Turner, 1997).
       Ventrally, V1 neurons express En1 (Ericson et al., 1997) and
30   Lim3$^+$ V2 neurons express Chx10 (Ericson et al. 1997).

       In MNR2-infected embryos D1, D2, V1 or V2 interneuron
       markers were not detected in ectopic Isl2$^+$ and HB9$^+$ neurons
       (data not shown). In regions of the spinal cord in which a
35   high proportion of cells in these interneuron domains
       ectopically expressed MNR2 there was a ~50-90% decrease in
       the number of cells that expressed D1, D2, V1 and V2 neuron
       markers (Table 1).

Table 1. Suppression of Spinal Interneuron Fates by MNR2

Interneuron Population	Homeodomain Protein Marker	Reduction in Marker Expression
D1	LH2	75% (1)
D2	Brn3.0	88 $\pm$ 4% (4)
V1	En1	46 $\pm$ 5% (10)
V2	Chx10	89 $\pm$ 3% (9)

Analysis derived from spinal cord of a stage 22 MNR2-infected embryo in which ectopic MNR2$^+$ cells were restricted almost exclusively to the right half of the spinal cord. Values (%) indicate number of labeled cells on heavily infected vs uninfected sides. Mean $\pm$SEM, n = number of sections. A similar repression of interneuron markers was detected in six other MNR2-infected embryos.

The differing degrees of extinction of these interneuron markers appear to reflect local variation in the density of ectopic MNR2$^+$ cells rather than any difference in the efficiency with which MNR2 suppresses individual interneuron fates. The induction of somatic motor neuron differentiation by MNR2 is therefore accompanied by the suppression of interneuron fates.

Cooperation of Isl1 and MNR2 in the Specification of Somatic Motor Neuron Identity

We next addressed the contributions of the transcription factors induced by MNR2 to the differentiation of somatic motor neurons. To examine the role of Isl1 in the program of somatic motor neuron differentiation we assayed motor neuron markers in embryos infected with Isl1 virus. No

ectopic expression of MNR2, Lim3, Isl2 or HB9 was detected (Fig 6 A-6E). Thus, <u>Isl1</u> is not sufficient to direct somatic motor neuron differentiation. Nevertheless, all MNR2-induced ectopic dorsal Isl2$^+$ and HB9$^+$ neurons coexpressed Isl1 (Fig 4E). This observation, together with the requirement for Isl1 in the generation of somatic motor neurons (Pfaff et al., 1996) suggested that the ectopic expression of Isl2 and HB9 induced by MNR2 involves Isl1.

To test this we analyzed the activity of MNR2 at an earlier stage of development (stage 20). We reasoned that if Isl1 cooperates with MNR2 in the induction of Isl2 and HB9, then the early onset of Isl1 expression by prospective D2 neurons (Liem et al., 1997) might relieve the delay inherent in the induction of Isl1 by MNR2 and thus accelerate MNR2-induced motor neuron differentiation. MNR2 induced ectopic dorsal expression of Isl2 and HB9 at stage 20 (Fig 6 F-6J). However there was a restriction in the position of ectopic Isl2$^+$ and HB9$^+$ cells, focused on the normal domain of D2 neuron generation (Fig 6 F-6J, 6P). If this restriction is conferred by the early expression of Isl1 by prospective D2 neurons then coinfection with <u>MNR2</u> and <u>Isl1</u> viruses would be expected to abolish the dorsoventral restriction in Isl2 and HB9 expression. In the spinal cord of embryos coinfected with <u>MNR2</u> and <u>Isl1</u> viruses and analyzed at stage 20, a high proportion of cells now coexpressed MNR2 and Isl1 at ectopic locations (Fig 6K). Over 70% of these cells coexpressed Isl2 and/or HB9 (Fig 6N). Moreover, the dorsoventral restriction in the position of ectopic Isl2$^+$ and HB9$^+$ neurons evident after <u>MNR2</u> infection was abolished (Figs 6 L, 6M, 6P). These findings support the idea that the induction of Isl2 and HB9 by MNR2 involves the cooperation of Isl1.

MNR2 Acts Upstream of Lim3 in the Specification of Somatic Motor Neurons

During normal somatic motor neuron differentiation, Lim3 and MNR2 are expressed by ventral progenitors at a similar developmental stage (Fig 2 J, 2K). To determine the

hierarchy of MNR2 and Lim3 activity, we misexpressed Lim3 and assayed the resulting pattern of homeodomain protein expression. Lim3 did not induce MNR2 or Isl2 (Fig 7 A-7E) nor did it increase the number of dorsal Isl1^{+} neurons (Fig

5   7D, 7E). However, there was a very low incidence of ectopic HB9^{+} neurons in the dorsal spinal cord of Lim3-infected embryos (Fig 7D, 7E) confined exclusively to cells that coexpressed Isl1 (Fig 7D; data not shown).

10   The restriction of ectopic HB9 expression to dorsal Isl1^{+} neurons in Lim3-infected embryos raised the possibility that the coordinate activities of Lim3 and Isl1 are sufficient to induce HB9 expression. To test this we coinfected embryos with Lim3 and Isl1 viruses (Fig 7H). The incidence of

15   ectopic expression of HB9 was markedly increased in the spinal cord of such coinfected embryos, compared to embryos infected solely with Lim3 (Fig 7 J, 7K). Moreover, > 30% of cells that coexpressed Lim3 and Isl1 expressed HB9 (Fig 7K) and these HB9^{+} neurons were not restricted to any

20   dorsoventral position (Fig 7J; data not shown).   In contrast, ectopic expression of Isl2 was rarely detected after Lim3 and Isl1 coinfection (Fig 7 I, 7K; data not shown). These results provide evidence that Lim3 and Isl1 cooperate as intermediaries in the MNR2-induced activation

25   of HB9 expression.

HB9 Activity and the Maintenance of Transcription Factor Expression by Somatic Motor Neurons

The expression of MNR2 is transient, raising the issue of

30   how the expression of Lim3, Isl1 and Isl2 is maintained in post-mitotic motor neurons.   The HB9 gene encodes a homeodomain protein closely related to MNR2, suggesting that HB9 has an activity similar to that of MNR2. To test this we infected embryos with an HB9 virus and monitored the

35   ectopic expression of Isl1, Isl2 and Lim3. HB9 induced the expression of Isl1, Isl2 and Lim3 at an efficiency similar to that of MNR2 (Fig 7 L-N; data not shown). Misexpression of HB9 also induced the ectopic expression of MNR2 (data not

shown), a result that we interpret to reflect mimicry of the
autoregulatory activity of MNR2. These results suggest a
role for HB9 in maintaining of the differentiated properties
of somatic motor neurons at times after MNR2 expression has
been extinguished.

**Lim3 Expression in the Absence of MNR2 is Sufficient to
Induce the V2 Interneuron Fate**

Lim3 is also expressed by V2 neurons and appears prior to
the V2 marker Chx10 (Ericson et al., 1997). We therefore
examined whether Lim3 has a role in the differentiation of
this interneuron subtype. To test this, we examined the
pattern of Chx10 expression in the spinal cord of Lim3-
infected embryos. Misexpression of Lim3 induced Chx10 in
Cyn1$^+$ neurons located at both dorsal and extreme ventral
regions of the spinal cord, adjacent to the floor plate (Fig
7 F, G; data not shown) but not in somatic motor neurons
(Fig 7G). These results suggest that the expression of Lim3
in cells devoid of MNR2 activity is sufficient to direct the
differentiation of V2 interneurons.

DISCUSSION

Graded Shh signaling appears to control the identity of
neuronal subtypes in the ventral neural tube. The present
studies show that Shh induces the expression of a
homeodomain protein, MNR2, in motor neuron progenitors and
that the expression of MNR2 is sufficient to direct somatic
motor neuron differentiation. Thus, MNR2 expression appears
to be a critical output of Shh signaling in the pathway of
somatic motor neuron generation. We discuss these findings
in the context of the Shh signaling pathways involved in
neuronal fate determination and the control of progenitor
cell identity and commitment in the vertebrate CNS.

MNR2 Expression Specifies Somatic Motor Neuron Progenitors
Shh signaling is required for the generation of somatic
motor neurons, but the downstream steps in this
developmental program have not been resolved. Somatic motor

neurons derive from Pax6^{+} progenitor cells, yet Pax6 itself
appears to be required only indirectly for somatic motor
neuron generation (Ericson et al., 1997). Our results
suggest that the expression of MNR2 by Pax6^{+} progenitors is
5   a key step in somatic motor neuron development, specifying
ventral cells as motor neuron progenitors.

At what step in the pathway of somatic motor neuron
differentiation does MNR2 act? MNR2 is expressed by ventral
10   progenitor cells ~4-5h prior to the generation of the first
post-mitotic motor neurons and the cell cycle time of
ventral progenitor cells is ~8h (Langman et al., 1966).
Thus, it appears that MNR2 expression is initiated during
the final division cycle of motor neuron progenitors. The
15   onset of MNR2 expression by motor neuron progenitors
coincides with the time that they attain independence of Shh
signaling (Ericson et al., 1996). This observation and the
ability of MNR2 to activate its own expression provide a
potential molecular basis for the transition of Shh-
20   dependent ventral progenitor cells into Shh-independent,
committed somatic motor neuron progenitors.

MNR2 is induced rapidly by Shh, prior to the expression of
other somatic motor neuron transcription factors. It is
25   unclear, however, if MNR2 is a direct target for the
conserved Hedgehog (Hh) signal transduction pathway mediated
by the Ci/Gli class of transcription factors (Ingham, 1995).
Gli proteins have been implicated in floor plate
differentiation (Lee et al., 1997; Hynes et al., 1997; Ding
30   et al., 1998; Matise et al., 1998) but it is uncertain
whether they are also involved directly in the generation of
motor neurons.

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MNR2 and the Transcriptional Hierarchy of Motor Neuron Differentiation

The expression of MNR2 in neural progenitor cells appears sufficient to induce somatic motor neurons. Moreover, the ability of MNR2 to direct motor neuron differentiation appears to be independent of the position of progenitor cells within the neural tube. Most strikingly, MNR2 promotes somatic motor neuron differentiation both in ventral progenitors that have been exposed to inadequate levels of Shh and in dorsal progenitor cells that have been exposed to BMP signals (Liem et al., 1997). Since exposure of progenitors to BMPs inhibits motor neuron generation (Basler, et al., 1993), these findings implicate MNR2 as a determinant of somatic motor neuron identity.

MNR2 appears to function upstream of a set of LIM homeodomain transcription factors that cooperate to specify somatic motor neuron identity (Fig 8). Lim3 is expressed soon after MNR2 in motor neuron progenitors. Ectopic expression of MNR2 induces Lim3 expression but Lim3 does not induce MNR2, suggesting that MNR2 functions upstream of Lim3 in motor neuron progenitors. However, Lim3 is also expressed by V2 neurons that appear to derive from MNR2⁻ progenitors, implying the existence of an MNR2-independent pathway for the activation of Lim3 expression. This parallel pathway could also operate in somatic motor neuron progenitors.

MNR2 efficiently induces the expression of Isl1 in spinal neurons. The induction of Isl1 may be a key step in somatic motor neuron differentiation, since the later appearance of transcription factors specific to somatic motor neurons, Isl2 and HB9, appears to require the expression of Isl1. The role of Isl1 inferred from these gain-of-function studies is consistent with the loss of somatic motor neuron differentiation in mice lacking Isl1 function (Pfaff et al., 1996). Isl1 is also required for the generation of visceral motor neurons (Pfaff et al., 1996). Since MNR2 is not

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expressed by Nkx2.2⁺ visceral motor neuron progenitors, the
expression of Isl1 in visceral motor neurons appears to be
regulated by a factor other than MNR2. A dorsal expansion
in the domain of Nkx2.2 expression results in a switch from
5   somatic to visceral motor neuron generation (Ericson et al.,
1997) suggesting that Nkx2.2 or related Nkx genes (Pabst et
al., 1998) promote visceral motor neuron identity.

Lim3 and Isl1 appear to cooperate in certain of the
10   downstream steps of somatic motor neuron differentiation,
their actions being sufficient to induce HB9 but not Isl2.
Indeed, biochemical evidence indicates that Isl1 and Lim3
can interact directly (Jurata et al., 1998). Different
intermediary pathways may therefore control the expression
15   of distinct components of the somatic motor neuron phenotype
(Fig 8). Although the coexpression of MNR2 and Isl1 can
induce Isl2 expression, Lim3 is induced by MNR2 and thus may
also participate in the induction of Isl2 expression. HB9
possesses an activity similar to that of MNR2 but appears
20   only in post-mitotic neurons and thus may function to
maintain somatic motor neuron properties after the
expression of MNR2 has been extinguished.

The expression of Lim3 in a cellular context devoid of MNR2
25   results in the activation of Chx10, a definitive marker of
V2 neurons. Lim3 many therefore function in the normal
program of generation of both somatic motor neurons and V2
neurons. Misexpression of MNR2 in the V2 interneuron domain
represses the expression of Chx10 while promoting somatic
30   motor neuron differentiation. Thus the restriction in the
domain of MNR2 expression imposed by graded Shh signaling
may underlie the decision of ventral progenitors to
differentiate into somatic motor neurons or V2 neurons.

35   **MNR2 Functions in Conjunction with an Independent Neurogenic
Program**
The activity of MNR2 in inducing somatic motor neuron
differentiation appears to operate within the context of a

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broader program of neurogenesis. Ectopic MNR2 induces the expression of Lim3 in progenitor cells but induces Isl1, Isl2 and HB9 only in post-mitotic neurons, consistent with the normal temporal appearance of these proteins in somatic motor neurons. Thus, neural progenitors appear unable to express post-mitotic somatic motor neuron markers even though MNR2 is expressed precociously in these cells.

The timing of differentiation of vertebrate neurons appears to be controlled by the sequential activation of basic HLH proteins with neurogenic properties (Anderson and Jan, 1997; Lo et al., 1998). The expression of certain of these bHLH genes is initiated at the time that progenitor cells exit the cell cycle and acquire overt neuronal character (Begley et al., 1992; Roztocil et al., 1997). An independently-regulated program of expression of neurogenic bHLH proteins might therefore limit the ability of MNR2 to induce the expression of Isl1, Isl2 and HB9 to post-mitotic neurons.

A Single Transcription Factor Specifies an Individual Neuronal Subtype

Our results provide some insight into the question of whether neuronal subtype identities in the developing vertebrate CNS are controlled by the actions of dedicated neuronal subtype-specific determinants. Studies of cell fate determination during vertebrate myogenesis and Drosophila eye development have revealed the existence of transcriptional cascades that can be activated by the expression of a single transcription factor (Weintraub, 1993; Halder et al., 1995; Chen et al., 1997; Pignoni et al., 1997). Our results indicate that one neuronal subtype generated in the vertebrate CNS, somatic motor neurons, can similarly be specified by the actions of a single homeodomain transcription factor, MNR2. Moreover, in a neuronal context devoid of MNR2 activity, Lim3 appears sufficient to specify V2 interneuron fates. The differentiation of floor plate cells can also be specified by a single transcription factor, the winged helix protein

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HNF3ß (Ruiz i Altaba et al., 1993; 1995; Sasaki and Hogan 1994).

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The identification of transcription factors that direct the generation of two distinct classes of neurons in the ventral spinal cord raises the possibility that the identity of neurons in other regions of the CNS are similarly determined by the activity of individual subtype-dedicated transcription factors. The identification of such factors

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might permit the direction of progenitor cells along specific pathways of neuronal differentiation in the absence of constraints imposed by their prior developmental history.

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SECOND SET OF EXPERIMENTS

Results

HB9 Expression in Developing Motor Neurons

To begin to define the role of HB9 in the early development
of MNs, we compared the pattern of HB9 expression with that
of three other early MN markers, the LIM-HD proteins Lim3
(Lhx3), Isl1 and Isl2.   In the mouse spinal cord and caudal
hindbrain, the first post-mitotic MNs are detected at e9.0
to e9.5 and MN genesis is complete by e10.5-e11.0 (Nornes
and   Carry,   1978;   Figure   16A;   our   unpublished   data).
Expression of HB9 was first detected in the spinal cord at
e9.25 to e9.5 (Figure 14; data not shown). During the early
phase of MN generation (when 10-25% of the total number of
MNs destined to form at a specific axial level have been
generated), the expression of HB9 was largely coincident
with that of Lim3 (Figure 14A; data not shown), and
HB9$^+$/Lim3$^+$ cells that lacked Isl1 expression could also be
detected (Figure 14E). However, during the peak period of
MN generation (25-60% of total MN number), many medially
located Lim3$^+$ cells lacked HB9 expression (Figure 14B-D) and
the expression of HB9 and Isl1 coincided in virtually all
cells (Figure 14F-H). At e10.0, near to the completion of
the period of MN generation (60-85% motor neuron genesis),
HB9 and Isl1 expression also coincided, but by this stage
many of the more lateral and thus more mature HB9 cells had
lost Lim3 expression (see Figure 17A, E).   Within the
developing spinal cord, HB9 expression was detected in all
MNs, regardless of their eventual somatic or visceral
subclass identity (data not shown). However, a more
selective profile of HB9 expression was detected at caudal
hindbrain levels. Here, HB9 expression was restricted to
somatic MNs of the hypoglossal nucleus and no expression was
detected in cranial level MNs of visceral identity (data not
shown, but see Briscoe et al., 1999). Notochord cells also
transiently expressed HB9, from e8.5 to e9. 5 (Figure 14 A-
D; data not shown).

Comparison of the patterns of expression of Lim3, HB9 and

Isl1 raised the issue of the relationship between the time
of onset of HB9 expression and the exit of MNs from the cell
cycle.   To assess this issue we determined over the period
e9.5 to e10.0, the incidence of overlap in expression of HB9
5   with MPM2, a marker of M phase cells (Westendorf et al.,
1994), and with cells labeled by a brief BrdU pulse. The
incidence of coexpression of MPM2 and HB9 by individual
cells varied markedly according to the fraction of total
number of MNs generated at particular axial levels of the
10   spinal cord. We focused our quantitative analysis of HB9 and
MPM2 expression at caudal cervical levels of the spinal
cord. The incidence of MPM2/HB9 coexpression was greatest in
younger (e9.5) embryos in which only 10-25% of the total
number of MNs had been generated (Figure 14I; data not
15   shown).   In slightly older (e9.75) embryos, when MN
generation was approaching its peak (25-60% of motor neurons
generated), very few MPM2/HB9 colabelled cells were detected
(Figure 14J-L). During the later phase (e10.0) of motor
neuron generation (>60% motor neurons generated), few if any
20   HB9 cells coexpressed MPM2 (data not shown).   BrdU labeling
analysis of e9.5 to e10.0 embryos revealed that at early
stages of motor neuron genesis, BrdU$^+$/HB9$^+$ cells could be
detected (Figure 14N), but during the peak and late periods
of motor neuron genesis, the number of BrdU$^+$/HB9$^+$ cells
25   decreased markedly (Figure 14 O, P).   In contrast, many
BrdU$^+$/Lim3$^+$ cells were detected in the ventral domain of
motor neuron generation throughout the entire period of
motor neuron genesis (Figure 14M; data not shown).   These
data indicate that HB9 is expressed by some Lim3$^+$ MN
30   progenitors during the early phase of MN genesis, but also
suggest that during the peak period of MN genesis, many
cells initiate HB9 expression after the onset of Lim3
expression and coincident with the onset of Isl1 expression,
apparently as they acquire a post-mitotic state.

35

Generation of Hb9 Mutant Mice

To examine the role of Hb9 in early MN development, we
generated targeted alleles of Hb9 by homologous
recombination in mouse ES cells. Three Hb9 alleles were
generated, each containing a pgk-Neo gene and cDNAs encoding
different marker proteins under the control of an IRES
sequence (Ghattas et al., 1991; Mombaerts et al., 1996)
(Figure 15 A, Bii-iv): i/ an SV40 nuclear localization
signal (nls) fusion with lacZ (Hb9^{nlslacZ}) (Figure 15 D), ii/
a tau fusion with lacZ (Hb9^{taulacZ}) (Figure 15E, F) and iii/ a
tau fusion with a multimerized myc epitope (Hb9^{taumyc}) (data
not shown; see Figure 18K). Mice homozygous for each of the
targeted alleles were born at normal Mendelian frequencies
(Table 1) but died at or soon after birth (data not shown).
The cause of death has not been defined but may involve
respiratory failure, since the lungs of Hb9 mutant neonates
were not inflated (data not shown).

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Table 1. Generation of Mutant Embryos

	Number of Litters	Number of Embroys	Genotype +/+	+/-	-/-
Hb9 nclazZ	14	88	27	40	21
Hb9 taumyc	6	44	12	22	10
Hb9taulacZ	6	41	9	22	10
Total	26	173	48	84	41

To determine whether the targeted Hb9 alleles represented null mutations, we examined the expression of HB9 in heterozygous and homozygous mutant embryos at e10.5. In the spinal cord of heterozygous Hb9^{nlslacZ} embryos, the expression of lacZ and HB9 coincided (Figure 15 F, G). In mice homozygous for the Hb9^{nlslacZ} allele, the number and position of lacZ cells was similar to that in heterozygotes, but no expression of endogenous HB9 was detected with either N- or C-terminally directed antisera (Figure 15 G,H; data not shown). Similar results were found in homozygous Hb9^{taulacZ} and Hb9^{taumyc} mutants (data not shown). These results indicate that the targeted Hb9 alleles represent null mutations and also that the integration of lacZ into the Hb9 locus does not perturb the pattern of gene expression. The spatial pattern of endogenous HB9 expression was also mimicked in transgenic mouse embryos that expressed -galactosidase (lacZ) under the control of a ~9kb 5' fragment of the mouse Hb9 gene (Figure 15A, Bi, C; data not shown; see Experimental Procedures).

The persistence of lacZ expression in Hb9^{nlslacZ} mutants indicates that the maintained expression of Hb9 does not require positive autoregulation. We noted, however, that the level of lacZ expression was much greater in homozygous than in heterozygous Hb9^{nlslacZ} embryos (data not shown). To

test whether this increase results from a function of HB9 in
the repression of its own expression we compared the level
of lacZ expression in Hb9$^{\text{taulacZ}}$ / Hb9$^{\text{taumyc}}$ mutant embryos with
that in Hb9$^{\text{taulacZ}}$ heterozygous embryos. The level of lacZ

5   expression was >5 fold greater in the compound mutants than
in Hb9$^{\text{taulacZ}}$ heterozygotes (Figure 15E,F), even though both
sets of embryos expressed only a single Hb9$^{\text{lacZ}}$ allele. This
result indicates that HB9 represses, directly or indirectly,
its own transcription.

10

**Motor Neurons are Generated in Normal Numbers in Hb9 Mutant
Mice**

The transient expression of HB9 by the notochord led us to
examine whether the loss of Hb9 function affects the

15   establishment of progenitor cell domains within the ventral
neural tube. At e10.5, the expression of Shh, Pax6 and
Nkx2.2 in the ventral spinal cord and hindbrain of
homozygous Hb9$^{\text{nlslacZ}}$ embryos was indistinguishable from that
in heterozygous or wild type littermates (data not shown).

20   Thus, Shh signaling and the establishment of appropriate
ventral progenitor cell domains appear to be unaffected in
Hb9 mutants.

We next examined whether the initial generation of post-

25   mitotic MNs is affected by the loss of Hb9 function. LacZ
expression was detected in the nuclei of cells in the
ventral spinal cord in homozygous Hb9$^{\text{nlslacZ}}$ embryos at a
position and number identical to that in heterozygotes
(Figure 15 G, H, 16A). The expression of lacZ persisted in

30   MNs throughout embryonic development in both heterozygous
and homozygous Hb9$^{\text{nlslacZ}}$ mutant embryos (Figure 16E, F; data
not shown). Thus, cells in Hb9 mutant embryos retain a MN
identity, as assessed by the transcription of Hb9 itself.
In addition, the total number of Isl1 MNs generated between

35   e9.5 and e10.5 was similar in heterozygous and homozygous
Hb9$^{\text{nlslacZ}}$ embryos (Figure 17E, F, G; data not shown).
Moreover, the expression of Choline Acetyltransferase (ChAT)
by MNs persisted in homozygous Hb9$^{\text{nlslacZ}}$ embryos (see Figure

19B, C; data not shown). Together, these results indicate that HB9 is not required for the initial specification of MN identity.

Defects in Motor Neuron Migration in Hb9 Mutant Embryos

To determine whether aspects of the differentiation of post-mitotic MNs are affected by the loss of Hb9 function we first examined whether the overall spatial organization of MNs within the spinal cord and caudal hindbrain was affected. A marked defect in the pattern of MN migration was detected in Hb9 mutants. In wild type and heterozygous Hb9nlslacZ embryos examined at e10-e11, all MN cell bodies are confined to the spinal cord (Figure 15D, F, G; data not shown). In contrast, in homozygous Hb9nlslacZ embryos, ~5% of lacZ neurons were detected outside the spinal cord, within the ventral roots (Figure 16B). These extraspinal lacZ neurons, like their counterparts within the spinal cord, coexpressed Isl1 and Isl2 (Figure 16C; data not shown). HB9 is one of the few transcription factors that distinguishes MNs from dorsal root ganglion (DRG) neurons, raising the possibility that the detection of extraspinal lacZ neurons in Hb9 mutants was a result of their conversion to a DRG neuron identity. However, ectopic lacZ neurons did not express the sensory neuron-specific POU domain protein Brn3.0 (Figure 16D), arguing against their sensory neuron character. These findings reveal a role for HB9 in confining the cell bodies of MNs to the spinal cord.

The settling pattern of the MNs that remained within the spinal cord and caudal hindbrain was also disrupted in Hb9 mutants. At caudal hindbrain levels in heterozygous Hb9nlslacZ embryos, somatic hypoglossal MNs form a well-delineated nucleus (Figure 16E). Similarly, at cervical and lumbar levels of the spinal cord a clear separation of median motor column (MMC) MNs and lateral motor column (LMC) MNs is normally evident (Figure 16G, I). In contrast, in homozygous Hb9nlslacZ mutants there was a marked dispersion of MNs in the region of the hypoglossal motor nucleus (Figure 16F) and no

clear separation of MMC and LMC neurons was evident at cervical and lumbar levels (Figure 16H, J). These results suggest that the migratory and settling patterns of MNs that underlie the formation of discrete somatic motor nuclei at cranial levels, and of the major somatic motor columns at spinal levels are disrupted in Hb9 mutants.

Persistent Expression of Lim3 and Gsh4 by MNs in Hb9 Mutants

In wild type embryos, the expression of the LIM-HD proteins Lim3 and Gsh4 is rapidly lost from most post-mitotic MNs (Tsuchida et al, 1994; Ericson et al., 1997; Sharma et al., 1998). To begin to address whether there are changes in the identity of MNs in Hb9 mutant mice, we examined the profile of expression of Lim3 and Gsh4.

At e10.5 the total number of Lim3 cells was increased ~2 fold in homozygous Hb9^{nlslacZ} embryos compared to heterozygous embryos (Figure 17A-F). Over 98% of lacZ cells in homozygous Hb9^{nlslacZ} embryos coexpressed Lim3, compared to 23% in heterozygous embryos (Figure 17A, B, D), indicating that the increase in Lim3 neurons results from persistent expression in MNs. The persistence of Lim3 expression was widespead, occurring in post-mitotic MNs at all spinal levels and in the hypoglossal motor nucleus at caudal hindbrain levels (data not shown). In contrast, the number and position of Lim3 progenitor cells (defined as medial Lim3$^+$, lacZ$^-$, Isl1$^-$ cells) was unaffected (Figure 17A, B; data not shown). The expression of Gsh4 by MNs was maintained in a manner similar to that of Lim3 (data not shown). These results reveal that HB9 is required for the rapid extinction of Lim3 and Gsh4 expression from most somatic MNs, soon after their exit from the cell cycle. However, the deregulated expression of Lim3 and Gsh4 by MNs in Hb9 mutant embryos was not sustained. By e12.5 to e13.5, the number of Lim3 cells in homozygous Hb9^{nlslacZ} embryos was only 10-15% greater than in heterozygous embryos (Figure 17C, H), and there was a corresponding decrease in the number of cells that coexpressed Lim3 and lacZ (Figure 17D). Thus, factors other than HB9 are

eventually sufficient to extinguish Lim3 and Gsh4 expression from most somatic MNs.

Motor Neurons Transiently Acquire V2 Interneuron Character in the Absence of Hb9 Function

What is the consequence of the persistent expression of Lim3 and Gsh4 by MNs? In wild type embryos, Lim3 progenitors give rise to both MNs and V2 interneurons (Ericson et al., 1997a), but V2 neurons can be distinguished by expression of the homeodomain protein Chx10 (Liu et al., 1994; Ericson et al., 1997a). Since Lim3 is sufficient to induce ectopic Chx10 expression in spinal interneurons (Tanabe et al., 1998), we reasoned that the persistence of Lim3 expression in homozygous Hb9^{nlslacZ} embryos might lead MNs to acquire properties characteristic of V2 interneurons. To test this, we examined the expression of Chx10 in homozygous Hb9^{nlslacZ} embryos.

The total number of Chx10 neurons was increased 2-3 fold in homozygous Hb9^{nlslacZ} embryos examined over the period e10.5 to e12.5 (Figure 18 A-F). To determine whether the increase in Chx10 neurons reflects expression in MNs, we examined the incidence of coexpression of Chx10 and lacZ. No coexpression of Chx10 and lacZ was detected in heterozygous Hb9^{nlslacZ} embryos, whereas in homozygous Hb9^{nlslacZ} embryos 50% of lacZ cells coexpressed Chx10 (Figure 18B, C) and many of these cells also expressed Isl1 (Figure 18E, F). In homozygous Hb9^{nlslacZ} embryos, MNs did not express En1, a marker of V1 neurons that derive from progenitors that do not express Lim3 (data not shown; Ericson et al., 1997a). Thus, the acquisition of interneuron properties by MNs appears specific to markers of V2 neuron identity. Since Lim3 and Gsh4 expression are eventually extinguished from somatic MNs in Hb9^{nlslacZ} mutants, we examined whether the ectopic expression of Chx10 was also transient. Ectopic Chx10 expression was lost from MNs over the period e10.5 to e13.5, whereas expression in V2 neurons persisted (Figure

18C,F; data not shown). Thus in the absence of Hb9 function, MNs maintain expression of Lim3 and Gsh4, albeit transiently, and apparently as a consequence, acquire expression of the V2 neuron marker Chx10.

The expression of Chx10 by MNs in Hb9 mutants raised the issue of whether these neurons still project axons in a MN-like trajectory or acquire the projection pattern of V2 neurons, a class of ipsilateral spinal projection interneurons (Sharma, Jessell and Pfaff, unpublished data). We therefore examined whether $Chx10^+$, $lacZ^+$, $Isl1^+$ neurons projected axons into the periphery in $Hb9^{nlslacZ}$ mutants. After injection of HRP into the proximal region of the developing limb in e11.5 homozygous $Hb9^{nlslacZ}$ embryos, many $Chx10^+$, $Isl1/2^+$ neurons were labeled (Figure 18H, J). In contrast, in heterozygous $Hb9^{nlslacZ}$ mutants, labeled $Isl1/2^+$, $lacZ^+$ MNs did not express Chx10 (Figure 18G, I). These results show that in Hb9 mutants, many neurons that express the V2 neuron marker Chx10 continue to project axons out of the ventral root and thus retain an axonal projection characteristic of MNs.

These findings do not exclude, however, that a fraction of the Chx10 MNs in homozygous $Hb9^{nlslacZ}$ embryos acquired an axonal trajectory characteristic of V2 neurons. To test this we analyzed parasagittal sections through the ventrolateral funiculus of the spinal cord of e12.5 heterozygous and homozygous $Hb9^{taumyc}$ embryos. We used the $Hb9^{taumyc}$ allele for this analysis since the intensity of labeling and discrimination of intraspinal axons was greater than with the $Hb9^{taulacZ}$ allele (data not shown). In both heterozygous and homozygous $Hb9^{taumyc}$ embryos, myc-labeled axons were detected in the ventral roots (Figure 18K, L), but myc-labeled intersegmental axons in the ventral funiculus were not detected (Figure 18M, N; data not shown). Thus, the ectopic expression of Chx10 by MNs appears not to be sufficient to reroute axons along an intraspinal trajectory.

The Emergence of MN Subtype Identity is Disrupted in Hb9 Mutants

The early alteration in migratory behavior and in the profile of transcription factor expression by MNs in Hb9 mutants prompted us to examine whether the later molecular programs that define aspects of MN identity are affected. We first examined whether the profile of expression of Isl1, a LIM-HD protein that is initially expressed by all post-mitotic MNs, is altered at later stages in Hb9 mutants.

Although the initial number of Isl1 MNs was not altered by the loss of HB9 function (Figure 17G), from e10.5 onwards there was a progressive decline in the number of Isl1 neurons. By e13.5 the number of Isl1 neurons detected in homozygous Hb9^{nlslacZ} embryos was only ~30% that in heterozygous or wild type embryos (Figure 17G). The loss of Isl1 expression was not the consequence of the death of MNs since no increase in TUNEL-labeled cells was detected in the ventral spinal cord over this period (data not shown), and the total number of lacZ cells was not reduced. Thus, despite the initial generation of MNs in normal numbers in homozygous Hb9^{nlslacZ} embryos, a marked disruption in the dynamic profile of expression of several LIM-HD proteins soon becomes evident.

By e12.5-e13.5, MNs have acquired distinct class, columnar and pool identities that can be defined by the expression of specific molecular markers (Figure 19A). We therefore examined whether the expression of MN subtype markers is affected by the loss of HB9 function.

MN Class Identity: The segregation of spinal MNs into somatic and visceral classes was maintained in Hb9 mutant embryos. Somatic MNs can be identified at e12.5 by expression of Isl2 (Tsuchida et al., 1994) and at this stage the number of Isl2 neurons was similar in homozygous and heterozygous Hb9^{nlslacZ} mutants (Figure 19D, E; data not shown). Thus, somatic MN differentiation occurs in the

absence of HB9 function.

In the spinal cord, visceral MNs are generated predominantly at thoracic levels and can be defined by their location within the intermediate region (Barber et al., 1991; Markham and Vaughn, 1991), and molecularly, by their expression of -nicotinamide adenine dinuclotide phosphate diaphorase (NADPH-d) activity (Wetts et al., 1995). The number of neurons in the intermediate spinal cord that expressed NADPH-d neurons was markedly reduced in Hb9^{nlslacZ} mutants examined at e14 to e17 (Figure 19F, G; data not shown). The loss of NADPH-d expression, however, was much more marked in medially located neurons and many laterally located NADPH-d MNs were still detected (Figure 19F, G; data not shown). The extent of ChAT expression by MNs in medial regions of the intermediate spinal cord was also significantly reduced in Hb9^{nlslacZ} mutants (Figure 19B, C; data not shown). LacZ neurons were, however, detected at approximately normal numbers within both the medial and lateral regions of the intermediate spinal cord in homozygous Hb9^{nlslacZ} embryos (data not shown), suggesting that the differentiation rather than the generation of visceral MNs is affected by the loss of Hb9 function. The generation and differentiation of visceral MNs at caudal hindbrain and cervical spinal cord levels was not affected in Hb9 mutant embryos, consistent with the finding that cranial visceral MNs do not express HB9 (Briscoe et al., 1999).

MN Columnar Identity: We next examined the expression of molecular markers that define the columnar subtype identity of MNs. By e12.5-13.5, Lim3 MNs are normally restricted to the medial MMC (Figure 19H) but in homozygous Hb9^{nlslacZ} embryos, Lim3 MNs were more widely dispersed throughout the LMC domain (Figure 19I). Although this result could simply reflect the persistence of expression of Lim3 by many MNs, it provides evidence that neurons with a molecular profile characteristic of the medial MMC are displaced in Hb9 mutants.

The differentiation of MNs within the LMC was also disturbed in Hb9 mutants. At limb levels of the spinal cord, the expression of <u>Retinaldehyde dehydrogenase 2</u> (<u>RALDH2</u>), a generic marker of LMC neurons (Zhao et al., 1996; Sockanathan and Jessell, 1998), was markedly reduced in homozygous <u>Hb9^{nlslacZ}</u> embryos (Figure 19J, K). Moreover, the differentiation of MNs within the lateral subdivision of the LMC, characterized by coexpression of Isl2 and Lim1 was also greatly reduced (Figure 19L, M). Nevertheless, some lateral LMC MNs were detected, suggesting that the columnar subdivision of the LMC is not completely abolished in <u>Hb9</u> mutants.

<u>MN Pool Identity</u>: Within the LMC, distinct motor pools can be distinguished by expression of the ETS proteins PEA3 and ER81 (Lin et al., 1998; Arber, Lin, Shneider and Jessell, unpublished data). A marked reduction in the expression of PEA3 and ER81 was detected in LMC MNs, both at forelimb and hindlimb levels. At forelimb levels, for example, a medial LMC-derived motor pool that innervates the pectoralis muscle expresses PEA3 (Figure 19N). In homozygous <u>Hb9^{nlslacZ}</u> embryos, PEA3 expression was almost completely absent from MNs at this axial level (Figure 19O). The disruption in expression of ETS protein expression was generally more severe than the loss of columnar markers (data not shown). ER81 and PEA3 expression by subsets of dorsal root ganglion neurons was, however, not affected in homozygous <u>Hb9^{nlslacZ}</u> embryos (Figure 19N, O; data not shown). Taken together, these results reveal that the elimination of HB9 function results, directly or indirectly, in a disruption in the establishment of the class, columnar and pool identities of MNs

Defects in Motor Axon Trajectories in Hb9 Mutant Mice

What are the consequences of the marked disruption in the molecular program of MN differentiation for the connectivity of MNs? To begin to address this issue, we examined whether the projection of motor axons towards their peripheral targets is altered in Hb9 mutant embryos.

In heterozygous Hb9^taulacZ embryos examined at e11.5, the pattern of axonally transported lacZ expression coincided with the trajectories of somatic MNs (Figure 20A; data not shown; see Ericson et al., 1997a). For example at hindbrain levels, the hypoglossal nerve, and at cervical spinal cord levels the segmentally-arrayed projections of somatic motor axons in the ventral roots, were delineated by lacZ expression (Figure 20 A). LacZ-labelled peripheral motor axons were also detected in homozygous Hb9^taulacZ embryos (Figure 20B-D; data not shown), indicating that HB9 is not required for the initial projection of motor axons into the periphery. However, there were marked, albeit variable, defects in the more distal projections of motor axons in homozygous Hb9^taulacZ embryos (Figure 20B-D; data not shown). One of the most dramatic instances of the variability of the defects in motor axon trajectory was detected at caudal hindbrain levels. The hypoglossal motor nerve was either absent completely or severely misrouted in some Hb9 mutant embryos (Figure 20B; data not shown), but present with an apparently normal trajectory in others (Figure 20C).

The disruption in motor axon trajectories in Hb9 mutants led us to examine whether the major branches of somatic motor axons to axial and limb regions were formed. Analysis of the pattern of motor nerve branching both in embryos stained in whole-mount and in transverse sections revealed a marked defect in the organization of motor nerves at the axial muscle branch point and at the plexus region at the base of the fore- and hind-limbs. In heterozygous Hb9^taulacZ and Hb9^taumyc embryos, motor axons at these plexus regions formed a tight fascicle (Figure 20A, E, F), whereas in homozygous embryos

motor axons were dispersed and defasciculated (Figure 20 B, C, G, H; data not shown). Nevertheless, motor axon projections to axial muscles were detectable in homozygous Hb9^{taulacZ} and Hb9^{taumyc} embryos (Figure 20E-H; data not shown). Similarly, distinct motor axon branches projected into the dorsal and ventral halves of the limb mesenchyme in Hb9 mutant embryos (Figure 20D, G). Thus, there are marked defects in the peripheral organization of motor nerves at sites of critical motor axon pathfinding decisions, although the major peripheral axon branches of somatic MNs are present.

Defects in Muscle Innervation by Motor Axons in Hb9 Mutants
The defect in the peripheral organization of motor nerve branches in Hb9 mutants led us to examine whether there are also defects in the innervation of target muscles. We first analysed the innervation of limb muscles in late embryonic stage (e17.5) embryos, using GAP-43 expression to visualize peripheral axons and -bungarotoxin labeling to delineate clusters of acetylcholine receptors (AChR) on the muscle surface. We detected no obvious defect in the pattern of motor innervation or in AChR expression in fore- and hind-limb muscles in Hb9 mutant embryos (data not shown).

Hb9 mutant mice die soon after birth with uninflated lungs, and we therefore considered the possibility that the innervation of the diaphragm muscle by phrenic MNs might be affected. In the diaphragm of wild type and heterozygous Hb9^{nlslacZ} neonates, AChR clusters were restricted to a central domain of the muscle that coincided with the position of muscle innervation (Figure 20I). In contrast, in homozygous Hb9^{nlslacZ} mice, AChR clusters were present but distributed over a much wider region of the muscle surface (Figure 20J). Many of these scattered AChR clusters were abnormally small (Figure 20J; data not shown), and the thickness of the diaphragm muscle was markedly reduced in homozygous Hb9^{nlslacZ} embryos (Figure 20K, L). In addition, in the diaphragm of

wild type and heterozygous Hb9^{nlslacZ} mice, AChR clusters were invariably associated with GAP43-labeled nerve terminals (Figure 20K). In contrast, in homozygous Hb9^{nlslacZ} mice, many AChR clusters were not associated with GAP43-labeled nerve branches (Figure 20L). Extensive axonal sprouting was also detected in regions of the diaphragm close to the point of entry of the phrenic nerve (data not shown). These observations reveal a marked defect in the innervation of certain skeletal muscles in Hb9 mutant embryos. They also raise the possibility that the perinatal lethality of Hb9 mutant animals results, at least in part, from the diaphragm denervation phenotype.

Discussion

This paper provides evidence that many aspects of the differentiation of post-mitotic MNs in the mammalian spinal cord depend on functions provided by the homeobox gene Hb9. Our findings add to an understanding of the molecular steps of MN differentiation in two main ways. They provide genetic evidence that the signals involved in the generation of MNs can be dissociated from the later steps that consolidate MN identity. In addition, they provide evidence that a transcription factor expressed at an early stage in the differentiation of all spinal MNs has an essential function in the acquisition of MN subtype identities. Thus, they provide an initial insight into the hierarchical relationship of the transcription factors that define distinct functional subsets of MNs in the developing spinal cord. We discuss the possible roles of HB9 in the progressive specification of MN identity that are suggested by this analysis of the Hb9 mutant phenotype.

A Requirement for HB9 Function in Early Motor Neuron Development

At the earliest stages of MN genesis, late-stage Lim3$^+$ MN progenitors express HB9. However, during the peak period of MN generation the onset of HB9 expression by most cells appears to occur after that of Lim3 and coincident with Isl1, apparently in newly generated post-mitotic MNs. Consistent with this, the loss of Hb9 function has no discernable effect on the number of spinal MNs initially generated. Thus, the phenotype of Hb9 mutant mice supports the idea that the relevant period of expression and function of HB9 is in post-mitotic MNs. Previous studies have analyzed MN differentiation in mice lacking Isl1, a LIM homeodomain protein expressed almost exclusively by post-mitotic MNs. However, in Isl1 mutant mice, MNs undergo rapid apoptotic death, (Pfaff et al., 1996; Ericson, Kania and Jessell, unpublished data), hindering an analysis of later steps in MN differentiation. Isl1 is initially expressed by MNs in Hb9 mutant mice but many neurons rapidly lose Isl1 expression. Despite this, MNs survive. This result, taken together with the more drastic MN phenotype of Isl1 mutant mice, suggests that MNs may require Isl1 for their survival only during a brief critical period, soon after cell cycle exit.

A Role for HB9 in the Consolidation of the Motor Neuron-V2 Interneuron Identity Decision

The progenitors of both MNs and V2 interneurons are marked by expression of Lim3 (and the functionally redundant protein Gsh4), implying that additional factors are required to select between these two neuronal fates. The analysis of changes in transcription factor expression by post-mitotic MNs in Hb9 mutant embryos provides support for the idea that emerged from studies in chick (Tanabe et al., 1998) that the MNR2/HB9 class of homeodomain proteins has a role in selecting MN rather than V2 interneuron identity within the ventral spinal cord. In the chick, ectopic expression of MNR2 and HB9 suppresses the generation of V2 interneurons

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and promotes MN differentiation, providing gain-of-function evidence that MNR2 and HB9 have such a neuronal subtype selector function. The present studies provide complementary loss-of-function evidence that HB9 serves such a function in

5   MNs, acting to repress the expression of V2 interneuron character and thus to consolidate MN fate (Figure 21). These results have the additional implication that the stabilization of MN rather than V2 interneuron identity may not be achieved until after MNs have left the cell cycle.

10

At a molecular level, the HB9-dependent consolidation of MN phenotype is likely to be mediated through the regulation of Lim3 (and Gsh4) (Figure 21). In Hb9 mutant embryos, the expression of Lim3 and Gsh4 persists in post-mitotic MNs for

15   an abnormally long period, implying that HB9 normally functions, directly or indirectly, to repress the expression of Lim3 and Gsh4. In the chick, ectopic expression of Lim3 is sufficient to induce V2 intrneuron markers in a cellular context in which MNR2 and other MN homeodomain proteins are

20   not expressed (Tanabe et al., 1998). It is likely, therefore, that the persistence of Lim3 and Gsh4 expression in Hb9 mutants is responsible for the ectopic expression of Chx10 by MNs (Figure 21). Nevertheless, MNs do not undergo a complete switch to a V2 interneuron identity in Hb9

25   mutants. The incomplete and transient nature of the switch to a V2 interneuron identity may reflect the prior action of progenitor cell factors that initiate the process of MN specification.

30   An important issue raised by our findings is that of the respective roles of HB9 in the development of mouse and chick MNs. In the chick, HB9 expression is restricted to post-mitotic MNs whereas MNR2 expression is detected at a much earlier stage, in MN progenitors (Tanabe et al., 1998).

35   In contrast, in mouse, HB9 expression is detected in some MN progenitors at early stages of MN genesis. But over the peak period of MN generation, our data provide evidence that the expression of HB9 is delayed with respect to that of

Lim3 and appears coincident with that of Isl1. Thus, HB9 appears to be expressed at a slightly earlier stage in the progression of MN differentiation in mouse than is its counterpart in chick. Nevertheless, the profile of expression of mouse HB9 does not include an early and prominent phase of expression in MN progenitors, a profile characteristic of chick MNR2. A mouse MNR2 homolog has not been isolated to date, however (C. William and Y. Tanabe; personal communication). Thus, we cannot exclude that in mouse, HB9 has subsumed the functions performed in chick by MNR2. If this is the case, the finding that MNs are generated in normal numbers in Hb9 mutants would argue for the existence of a MNR2/HB9-independent pathway of MN generation. As discussed previously (Tanabe et al., 1998), such a pathway must operate at least in the hindbrain since neither mouse nor chick cranial visceral MNs, nor their progenitors, express MNR2 or HB9 (Tanabe et al., 1998; Briscoe et al., 1999). The ability of MNR2 and HB9 to induce a coordinate program of MN differentiation in chick spinal cord, together with the MN phenotype detected in Hb9 mutant mice does, however, support the idea that this homeodomain protein subfamily has an important, if as yet incompletely defined, role in early MN differentiation.

The Acquisition of Motor Neuron Subtype Identities is Impaired in Hb9 Mutant Mice

The early post-mitotic period of MN development is accompanied by the differentiation of functional subsets of MNs that can be defined both in terms of their anatomical organization and by expression of specific molecular markers (see Figure 19A). Many aspects of the differentiation of distinct MN subtypes, including columnar divisional and pool identities, are markedly affected by the loss of Hb9 function (Figure 21).

These defects in MN subtype identity could simply be an indirect consequence of the persistence of expression of Lim3 and Gsh4 and/or the ectopic expression of Chx10.

Nevertheless, defects in the columnar and pool identities of
MNs are evident well after the phase of deregulation of
Lim3, Gsh4 and Chx10 expression has subsided. Thus, it
remains possible that HB9 has a more direct role in the
5   specification of MN subtype identity. HB9 could, for
example, function as a cofactor in a series of independent
molecular programs that regulate the emergence of columnar,
divisional and pool subtype identities. Alternatively, HB9
could act at an early point in an obligate sequential
10   program of somatic MN differentiation that progresses from
columnar to divisional to pool identity. One line of
evidence that argues against a strict requirement for
sequential programs of MN subtype differentiation derives
from the observation that retinoids appear able to impose a
15   lateral LMC character on thoracic level MNs that have not
acquired a generic LMC character, (Sockanathan and Jessell,
1998). Thus, MN divisional character, at least, may be
acquired independently of columnar character.

20   The proposed roles of the MNR2/HB9 proteins in regulating of
MN subtype identity may have some parallels with the
functions of the Phox2a/b homeodomain proteins in the
specification of sympathetic neuronal fate (Goridis and
Brunet, 1999; Lo et al., 1999; Pattyn et al., 1999). Phox2
25   proteins are necessary for the induction of transmitter
synthetic enzymes and other features of sympathetic
neuronal differentiation. In addition, at hindbrain levels,
the Phox2 proteins are expressed selectively by visceral
MNs, in a pattern complementary to that of MNR2 and HB9
30   (Goridis and Brunet, 1999; Briscoe et al., 1999). It is
possible, therefore, that the segregated expression of these
two families of homeodomain proteins contributes to
distinctions in somatic and visceral subtype identity of
cranial MNs.

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Perturbed Motor Neuron Migration and Axonal Projections in Hb9 Mutants

The loss of Hb9 function results in marked defects in the migratory and settling patterns of spinal MNs. The most striking migratory defect in Hb9 mutants is the failure of many MN cell bodies to be retained within the spinal cord. These defects in MN migration are accompanied by marked errors in the pattern of axonal projections of somatic MNs. There is, however, variability in the precise nature of the errors in motor axon projection between mutant embryos. One consistent axonal projection phenotype in the spinal cord of Hb9 mutant mice is a pronounced defasciculation of axons, evident at the branch point of axial and limb-directed motor axons, and also at the plexus region at the base of the limbs. This finding raises the possibility that adhesive interactions between motor axons and/or the ability of motor axons to respond to extrinsic guidance cues are impaired in Hb9 mutants.

Despite these defects, each of the major axonal branches of spinal somatic MNs, to axial muscles and to dorsal and ventral limb muscles, is present in Hb9 mutant embryos. The detection of overtly normal projections to axial muscles is not surprising since these axons originate from medial MMC neurons that normally retain expression of Lim3 and Gsh4 (Tsuchida et al., 1994; Ericson et al., 1997; Sharma et al., 1998). The detection in Hb9 mutant embryos of both dorsally and ventrally directed motor axon branches in the limb may reflect, in part, the residual differentiation of some lateral LMC neurons. Nevertheless, the axons of many MNs that have lost their LMC identities also appear able to make either dorsal or ventral projections into the limb bud. This finding is consistent with previous studies showing that thoracic level MNs that are committed to a MMC identity form dorsal and ventral axonal projections in the limb when transplanted to limb levels (O'Brien et al., 1990; O'Brien and Oppenheim, 1990; Ensini et al., 1998). It seems possible, therefore, that the acquisition of LMC divisional identity

provides MNs with the ability to select appropriately a dorsal or ventral trajectory, rather than embarking along one or other trajectory at random. The necessity for motor axons to project along either a dorsal or ventral path in the limb may reflect the presence of a non-permissive environment for axons in the central core of the developing limb mesenchyme (see Tosney and Landmesser, 1985).

Taken together, our studies provide evidence that HB9 has an essential role in consolidating the identity of spinal MNs and in particular in suppressing the expression of V2 interneuron character. Since HB9 expression persists in adult MNs it is possible that its activities help to maintain the differentiated properties of MNs into post-natal and adult life. Defining the direct downstream targets of HB9 may help to clarify further how the molecular and functional properties of MN subtypes are established.

Experimental Procedures

Generation of Hb9 Mutant and Transgenic Mice

Mouse genomic clones used in the generation of Hb9 mutant mice and transgenic lines were derived from a 129/Sv genomic library (Stratagene). The transgenic TgN(Hb9)SAX16 line was derived from a construct that used a 9kb NotI fragment comprising the 5' upstream region of the Hb9 gene inserted 5' to an IRES-nls-lacZ cassette (see below). The targeting vector for the production of Hb9 mutant mice was constructed from genomic fragments derived from a 9kB NotI fragment 5' to the first exon of Hb9 and a HindIII clone overlapping with the 5' clone, but extending into the 3' region of Hb9. A 6kB 5' Sse83871-NotI fragment spanning into the first exon of Hb9 and an adjacent 3kB 3' NotI-XbaI fragment were cloned into a vector containing a PmeI site for linearization of the targeting constructs. NotI or PacI flanked targeting cassettes were generated independently (and comprised IRES-nls-lacZ, IRES-tau-lacZ, IRES-tau-myc, a SV40 polyadenylation signal and a lox flanked PGK-NEO cassette). These cassettes were integrated into the basic targeting

construct. For the generation of expression cassettes, marker genes were inserted downstream of an internal ribosome entry site (IRES) from encephalomyocarditis virus (Ghattas et al., 1991), in frame with the ATG of a NcoI site
5 at the 3' end of the IRES (Mombaerts et al., 1996). Linearized targeting constructs were electroporated into W95 ES-cells, selected with G418 and screened for potential recombinants by Southern blot analysis. A 1kB 3' XbaI-XhoI fragment was used as a probe to screen for ES-cell
10 recombinants (SalI/XhoI digest). This probe generates a 20kB wild-type band and a 6kB mutant band. The average frequency of recombination for the four constructs described in this study was ~1:20. Recombinant clones were injected into C57BL/6J blastocysts to generate chimeric founders that
15 transmitted the mutant allele. All experiments presented in this study involved analysis of embryos derived from heterozygous 129Sv x C57BL/6J intercrosses.

A 5' Fragment of the Hb9 Gene Confers Motor Neuron-Specific
20 **Transgene Expression.**
A ~9kb Not1 fragment of Hb9 (Figure 15A, B i) was sufficient to direct a SV40-nlslacZ fusion protein to developing MNs in transgenic mouse embryos, examined at e10 (Figure 15 C; data not shown). The pattern of lacZ corresponded closely to the
25 expression of endogenous HB9 (Figure 14; 15C and data not shown). The restriction of transgene expression to somatic MNs was apparent at this stage at caudal hindbrain levels where lacZ, like HB9 was expressed by somatic hypoglossal MNs but was excluded from visceral vagal MNs (Briscoe et
30 al., 1999; data not shown). At sacral levels of e9.5-e10.5 embryos both endogenous HB9 expression and lacZ expression in transgenic mice was detected throughout the neural tube, in the notochord and also in the primitive gut tube (data not shown).
35

In Situ Hybridization, Histochemistry and Immunocytochemistry
For in situ hybridization analysis, sections were hybridized

with mouse <u>RALDH2</u> and <u>ChAT</u> specific digoxigenin-labeled
probes (Schaeren-Wiemers and Gerfin-Moser, 1993).
Antibodies used in this study were: monoclonal IgG anti-Hb9
(N-terminal), rabbit anti-HB9 (C-terminal; kindly provided
by S. Pfaff), Pax6, MPM2, NF160, BrdU-FITC (Tanabe et al.,
1998; Ericson et al., 1997), skeletal -actinin (Sigma),
anti-Lim1/2 (Tsuchida et al., 1994); rabbit anti-Lim3,
Isl1/2, Isl2, Hb9 (N-terminal), Nkx2.2, Chx10, Brn3.0
(Tanabe et al., 1998; Ericson et al., 1997), GAP-43 (Aigner
et al., 1995), PEA3 (Arber et al., unpublished, generated
against the 14 C-terminal a.a. of mouse PEA3 coupled to KLH,
used at 1:1000), myc (S. Morton and T.M. Jessell,
unpublished, generated against the myc epitope (Evan et al.,
1985) coupled to KLH, used at 1:8000), -Galactosidase
(Cappel); goat anti- -Galactosidase (Arnel), HRP (Jackson
labs); guinea pig anti-Isl1 (Tanabe et al., 1998). Cryostat
sections were processed for immunohistochemistry as
described (Tsuchida et al., 1994) using fluorophore-
conjugated secondary antibodies (Jackson Labs) (1:500 to
1:1000). Rhodamine labeled -bungarotoxin (Jackson Labs) was
used at 1:2000. Images were collected on a BioRad MRC 1024
confocal microscope.

BrdU Labeling and Histology

BrdU experiments were performed by intraperitoneal injection
of BrdU (Sigma, 50 g/g body weight) 2 h before sacrifice.
Detection of BrdU was performed as previously described
(Sockanathan et al., 1998). TUNEL assays were performed
using a kit from Boehringer, NADPH-diaphorase reactions were
performed as described (Wetts et al., 1995), -Galactosidase
whole-mount staining was performed as described (Mombaerts
et al., 1996).

Retrograde Neuronal Labeling

20% HRP (Boehringer), 1% Isolecithin (ICN) in PBS was
injected into the limb of e11.5 mouse embryos or into the
ventral funiculus of e13.5 spinal cord and incubated for 2-
4h as described (Landmesser, 1978b) before fixation and

immunocytochemical detection of HRP.

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THIRD SET OF EXPERIMENTS

During mouse embryonic development, Hb9 expression is first
evident at the approximately 8-somite stage (E8) in the
5   notochord, in the entire dorsal gut endoderm and in the
ventral endoderm at the prospective pancreatic level
(Fig.22a, and data not shown). In contrast, Pdxl at this
stage is expressed only in the ventral pancreatic endoderm
(Fig. 22b). Thus, in the dorsal pancreatic anlage, the
10   initiation of Hb9 expression appears to precede that of
Pdxl, whereas in the ventral anlage both genes are expressed
concurrently. By E9.5 the level of Hb9 protein is reduced
in both pancreatic buds and notochord (Fig. 22c, and data
not shown). At E10.5, only a low level of Hb9 expression
15   remain in the dorsal pancreatic bud and virtually no
expression is detected in the ventral bud or in the
notochord (Fig. 22e, and data not shown). In contrast, Pdxl
expression is maintained in both pancreatic buds at theses
stages (Ohlsson et al. 1993; Ahlgren et al. 1996) (Fig.
20   22d,f). Thus, cells in both the dorsal and ventral buds
express Hb9 transiently at early embryonic stages. At later
stages, Hb9 expression reappears in the differentiating
pancreas. At E17.5, Hb9 expression was detected in the
insulin-producing ß-cells (Fig. 22g) but not in glucagon-
25   producing α-cells (Fig. 22h) or somatostatin-producing δ-
cells (Fig. 22i). No Hb9 expression was observed in non-
endocrine pancreatic cells Fig. 22, and data shown). The ß-
cell-specific expression persisted in the adult pancreas
(data not shown). These results show that Hb9 is expressed
30   in two distinct developmental phases, first during the
evagination of the pancreatic buds and later in
differentiating ß-cells.

To determine the role of Hb9 in the development of the
35   pancreas, we analyzed mice in which Hb9 had been inactivated
by homologous recombination in ES cells (Arber et al.
submitted for publication). E17.5 Hb9^{nlslacZ} mutant embryos
exhibited an overtly normal gastrointestinal tract, although

the duodenum appeared expanded (Fig. 23a). Analysis of the pancreas revealed that the dorsal pancreatic epithelial bud was absent, whereas the ventral bud was present (Fig. 23a). The spleen, an organ that develops from the dorsal
5   mesenchyme, was present in Hb9 mutants (Fig. 23a), and analysis of B13.5 embryos showed that the mesenchyme that normally surrounds the dorsal pancreas was present and appeared to have developed normally (Fig. 23b). The lack of the dorsal pancreatic bud, therefore, appears to reflect a
10   selective defect in the specification and development of the presumptive dorsal pancreatic epithelium.

To determine the stage at which the development of the dorsal pancreas is perturbed, we analyzed E9.5-10.5 $Hb9^{nlslacZ}$
15   homozygous embryos by whole-mount detection of ß-galactosidase (ß-gal) activity. At E9.5, ß-gal activity was observed throughout the dorsal endoderm of both heterozygous and homozygous $Hb9^{nlslacZ}$ embryos (Fig. 23c,d). In homozygous $Hb9^{nlslacZ}$ embryos, the evagination of the dorsal pancreatic
20   bud failed to occur (Fig. 23c,d). Analysis of E9.5 $Hb9^{nlslacZ}$ heterozygous and homozygous embryos revealed a complete lack of Pdxl expression in the presumptive dorsal pancreatic epithelium of $Hb9^{nlslacZ}$ mutant embryos, whereas Pdxl expression in the ventral pancreatic epithelium appeared
25   normal (Fig. 23e,f). By E10.5, distinct dorsal and ventral pancreatic buds were visible in heterozygous embryos (Fig. 23g,h) but no dorsal bud was discernible in homozygous $Hb9^{nlslacZ}$ embryos (Fig. 23g,h). Transverse sections of E10.5 $Hb9^{nlslacZ}$ mutant embryos suggest that the prospective dorsal
30   bud instead remains a part of the developing duodenum (Fig. 23i,j). These results suggest that Hb9 is required for the evagination and subsequent development of the dorsal pancreatic bud.

35   To analyze the state of differentiation of the presumptive dorsal bud of $Hb9^{nlslacZ}$ homozygous embryos, we examined the expression of early pancreatic markers in E9.5-10.5 mutant embryos. In E9.5 wild-type embryos, Isl1 (Fig 24a), Nkx2.2

(Fig. 24c) and glucagon (Fig. 24e) were expressed in the dorsal pancreatic epithelium. Mesenchymal expression of Isl1 in Hb9^{nlslacZ} mutant embryos was indistinguishable from that of wild-type littermates (Ahlgren et al. 1997) but we failed to detect Isl-exprssing epithelial cells in the dorasal primordium (Fig. 24a,b). Similarly, Nkx2.2- and glucagon-positive cells were absent from the dorsal pancreatic anlage (Fig. 24a-f). In contrast, the Hb9 expression, as well as expression of early pancreatic markers, is detected in a normal temporal and spatial manner in the dorsal pancreatic bud of Pdx1$^{-/-}$ embryos (Ahlgren et al. 1996 and data not shown). These results provide evidence that loss of Hb9 function impairs the initial stages in the dorsal pancreatic program, blocking both epithelial evagination and differentiation of early pancreatic cell types. They also show that the arrest in dorsal pancreatic bud development in the Hb9^{nlslacZ} mutant mice is more severe than that observed in Pdx1$^{-/-}$ mice.

The signaling molecule Sonic hedgehog (Shh) has been shown to exert an inhibitory action on pancreatic development (Apquelvist et al. 1997; Hebrok et al. 1998; Kim et al. 1997). We therefore investigated whether the impairment in initiation of dorsal pancreatic development might result from the ectopic expression of Shh or Indian hedgehog (Ihh: Apquelvist et al. 1997; Bitgood et al. 1995) in the presumptive pancreatic epithelium. The expression of both Shh an Ihh was examined in E9.5 homozygous Hb9^{nlslacZ} embryos. Neither the level nor the domain of expression of Shh or Ihh was changed in the pancreatic epithelium of mutant embryos (Fig. 24q-j). Thus, the block in the specification of the dorsal pancreatic differentiation program in Hb9^{nlslacZ} mutants appears not to be a result of altered Shh or Ihh expression.

The presence of ventral pancreatic tissue as assessed in E17.5 Hb9^{nlslacZ} homozygous mutants indicates that the early development of the ventral pancreas is independent of Hb9

function. To analyze whether there is a later defect in the development of ventral pancreatic tissue, we examined the expression of the endocrine hormones insulin (Ins; Fig. 25a-d,g,h), glucagon (Glu; Fig. 25a,b), somatostatin (Som; Fig. 25c,d) and the exocrine enzymes amylase (Fig. 25e,f) and carboxypeptidase A (data not shown). Each of these markers was expressed in the ventral pancreas of Hb9^{nlslacZ} homozygous neonates and the amount and general organization of endocrine cells into islet-like clusters and of exocrine cells into acinar-like structures appeared normal (Fig. 25a-f). Double immunohistochemical analysis, however, revealed an approximately 20% decrease in the number of Ins-positive cells and an approximately 3-fold increase in the number of Som$^+$-cells (Fig.25c,d). Ins$^+$/Isl1$^+$ cells make up approximately 68% in wild-type and 53% in Hb9^{nlslacZ} homozygous neonates; Som$^+$/Isl$^+$ cells make up approximately 9% in wild-type an 25% in Hb9^{nlslacZ} homozygous neonates. The number of Glu-positive cells appeared unchanged in Hb9 mutant mice; Glu$^+$/Isl1$^+$ cells make up approximately 23% in both wild-type and Hb9^{nlslacZ} homozygous neonates (Fig. 25a,b).

We also noted a defect in the spatial organization of endocrine cells in individual islets. Thus, although islet cell clusters were present in the ventral pancreas of Hb9 mutants, they failed to organize themselves into the typical structure of maturing islets in which α-cells are located at the periphery and surround a core of ß-cells (Fig. 25a-d). The Ins-positive cells present in the ventral pancreas of Hb9 mutants still expressed the transcription factors (Edlund et al. 1998) Pdxl (Fig. 25g,h) and Nkx6.1 (data not shown) as well as glucokinase (data not shown). In contrast, the expression of glucose transporter type 2 (Glut2) was virtually undetectable (Fig. 25i,j). These histological analyses indicate that the ventral pancreatic epithelium generates both exocrine and endocrine cells in the absence of Hb9 function. These results also provide evidence that although the genesis of pancreatic ventrally derived ß-cells is independent of Hb9, the terminal

differentiation and maturation of ß-cells requires <u>Hb9</u>
function.

Our study provides further insight into the molecular events
that control the specification of pancreatic cell fate. Our
results show that <u>Hb9</u> is required selectively for specifying
the initial stages of dorsal pancreatic development. As a
consequence, the dorsal pancreatic bud does not develop in
<u>Hb9</u> mutant mice. In contrast, the ventral pancreatic
epithelium develops and generates both exocrine and
endocrine cell types, thus revealing an early molecular
distinctions between the programs for dorsal and ventral
pancreatic development. Nevertheless, the ventral pancreas
of <u>Hb9</u> mutant embryos exhibits a more subtle perturbation in
ß-cell differentiation and islet cell organization,
indicating a later role for <u>Hb9</u> in the development of
pancreatic ß-cells.

The development of the pancreas begins with the dorsal and
ventral protrusion of a region of the primitive gut
epithelium (Wessells et al. 1967; Pictet et al. 1972; Slack
et al. 1995; Edlund et al. 1998. Several homeodomain and
basic-helix-loop-helix class transcription factors, notably
Isl1, Nkx2.2, Pax4, Pax6, and NeuroD/2, have been shown to
exert important functions in the control of pancreatic
endocrine cell differentiation (Ahlgren et al. 1997; Naya et
al. 1997; Sander et al. 1997; Sosa-Pineda et al. 1997; St.
Onge et al. 1997; Sussel et al. 1998). These factors are
expressed at early stages of pancreas development but in
their absence, the initial steps of pancreatic development
proceed normally resulting only later in a perturbation of
the differentiation of pancreatic endocrine cell types
(Slack et al. 1995). The basic-helix-loop-helix class
transcription factor p48, in contrast, is required for
exocrine but not endocrine differentiation (Krapp et al.
1998).

The homeodomain protein IPF1/PDX1 is required at an earlier stage in pancreatic development. Mice and humans lacking Ipf1/Pdx1 lack a pancreas (Ahlgren et al. 1996; Jonsson et al. 1994; Offield et al. 1996; Stoffers et al. 1997).

Ipf1/Pdx1 appears nevertheless to act at a step downstream of the initial specification of the gut endoderm to a pancreatic fate (Ahlgren et al. 1996; Offield et al. 1996) These findings imply the existence of additional genes involved in the regulation of earlier stages of pancreatic development. Thus, despite increasing information on the identity and function of factc·s involved in pancreatic cell differentiation, the molecular steps that occur prior to the onset of Ipf1/Pdx1 function to specify early stages in the program of pancreatic differentiation are poorly defined. It also remains unclear how the initially separate (Pictet et al. 1972; Slack et al. 1995; Edlund et al. 1998) programs of dorsal and ventral pancreatic bud development are co-ordinated to produce a convergent developmental program. In this paper we report that in mouse embryos the homeobox gene Hb9 (Harrison et al. 1994) is expressed by cells in early developing dorsal and ventral pancreatic buds and later in insulin-producing -cells. Dorsal pancreatic development is blocked in mice lacking Hb9 function, but the ventral pancreas does develop and contains both endocrine and exocrine cells. However, the relative proportions and spatial organisation of the various endocrine cells in the ventral pancreas are perturbed. Thus, the requirement for Hb9 in pancreatic development reveals a molecular distinction in the dorsal and ventral differentiation programs and sequential actions at both early and late stages of pancreatic differentiation.

Results.

HB9 is expressed in two distinct phases of pancreatic development.

The homeobox gene Hb9 was initially isolated from a human B cell library (Harrison et al. 1994) During mouse embryonic development, the expression of HB9 is first evident at the

~8 somite stage (e8) in the notochord, in the entire dorsal
gut endoderm and in the ventral endoderm at the prospective
pancreatic level (Fig.22a and data not shown). In contrast,
at this stage IPF1/PDX1 is expressed only in the ventral
5   pancreatic endoderm (Fig. 22b). Thus, in the early dorsal
pancreatic anlage the initiation of HB9 expression appears
to precede that of IPF1/PDX1 expression, whereas in the
ventral   anlagen   HB9   and   IPF1/PDX1   are   expressed
concurrently. By e9.5 the level of expression of HB9 is
10   reduced in both the dorsal and ventral pancreatic buds, and
also in the notochord (Fig. 22c and data no`. shown). By
e10.5, only a low level of HB9 expression remains in the
dorsal pancreatic bud and virtually no HB9 expression is
detected in the ventral bud or in the notochord (Fig. 22e
15   and data not shown). In contrast, expression of IPF1/PDX1
is maintained in both pancreatic buds at these stages
(Ohlsson et al. 1993; Ahlgren et al. 1996) (Fig. 22d,f).
Thus, HB9 is expressed transiently at early stages by cells
in both the dorsal and ventral buds, and endodermal
20   expression of HB9 is down regulated prior to that of
IPF1/PDX1.

At late embryonic stages, however, HB9 expression reappears
in the differentiating pancreas. At e17.5, HB9 expression
25   was detected in the insulin producing -cells (Fig. 22g) but
not in glucagon producing -cells (Fig 22h) or in
somatostatin producing -cells (Fig. 22i). No expression of
HB9 was observed in non-endocrine pancreatic cells (Fig. 22
and data not shown). The -cell specific expression of HB9
30   persisted in the adult pancreas (data not shown). Together,
these results show that HB9 is expressed in two distinct
developmental phases, first during the evagination of the
pancreatic buds and later in differentiating -cells.

35   **Hb9-deficient mice lack a dorsal pancreas.**
To determine the role of Hb9 in the development of the
pancreas we analysed mice in which the Hb9 gene has been
inactivated by homologous recombination in ES cells. The -

galactosidase gene, placed downstream of an internal ribosome entry site (IRES), was inserted out-of-frame into the first coding exon of HB9 (Arber et al. submitted for publication) HB9nlslacZ heterozygous offspring survived to adulthood, were fertile, and did not exhibit any overt abnormalities. Hb9nlslacZ homozygous mice survived fetal development but died soon after birth, possibly from respiratory failure (Arber et al. submitted for publication). To investigate the state of pancreatic differentiation in mice lacking Hb9 function we initially examined e17.5 Hb9nlslacZ mutant embryos. These embryos exhibited an overtly normal gastrointestinal tract although the duodenum appeared expanded (Fig. 23a). Analysis of the pancreas, however, revealed that the dorsal pancreatic epithelial bud was completely absent whereas the ventral bud was present (Fig. 23a). The spleen, an organ that develops from the dorsal mesenchyme was present in Hb9 mutants (Fig. 23a) and analysis of younger, e13.5 embryos, showed that the mesenchyme that normally surrounds the dorsal pancreas was present and appeared to have developed normally (Fig. 23b). Thus, the lack of the dorsal pancreatic bud appears to reflect a selective defect in the specification and development of the presumptive dorsal pancreatic epithelium.

To begin to determine the stage at which the development of the dorsal pancreas is perturbed, we analysed e9.5-10.5 Hb9nlslacZ homozygous embryos by whole-mount detection of lacZ activity. At e9.5, lacZ activity was observed throughout the dorsal endoderm of both heterozygous and homozygous Hb9nlslacZ embryos (Fig. 23c,d). The lacZ-activity observed in the Hb9nlslacZ homozygous embryos provides evidence that the expression of Hb9 is not positively auto-regulated. One potential explanation for the increase in lacZ expression in homozygous mutants is that HB9 negatively controls its own gene expression. Alternatively, the increased lacZ-expression in the homozygous embryos may be due to expression from the additional copy of the lacZ allele. In favour of the former

possibility, analysis of Hb9 expression in the spinal cord has revealed that Hb9 gene expression in motor neurons is negatively auto-regulated(Arber et al. submitted for publication).

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In homozygous Hb9nlslacZ embryos the evagination of the dorsal pancreatic bud failed to occur (Fig. 23c,d). Analysis of e9.5 Hb9nlslacZ heterozygous and homozygous embryos revealed a complete lack of IPF1/PDX1 expression in the presumptive dorsal pancreatic epithelium of Hb9nlslacZ mutant embryos, whereas IPF1/PDX1 expression in the ventral pancreatic epithelium appeared normal (Fig. 23e,f). By e10.5, distinct dorsal and ventral pancreatic buds were clearly visible in heterozygous embryos (Fig. 23g,h) but no evidence of a dorsal bud was discernible in the homozygous Hb9nlslacZ embryos (Fig. 23g,h). Transverse sections of e10.5 Hb9nlslacZ mutant embryos suggest that the prospective dorsal bud instead remains a part of the developing duodenum (Fig. 23i,j). These results suggest that Hb9 is required for the evagination and subsequent development of the dorsal pancreatic bud.

Hb9 is required for initiation of dorsal pancreatic differentiation.

To analyse the state of differentiation of the presumptive dorsal bud of Hb9nlslacZ homozygous embryos we examined the expression of early pancreatic markers in e9.5-10.5 mutant embryos. In e9.5 wild-type embryos, Isl1 (Fig. 24a), Nkx2.2 (Fig. 24c), and glucagon (Fig. 24e) were expressed in the dorsal pancreatic epithelium. Mesenchymal expression of Isl1 in Hb9nlslacZ mutant embryos was indistinguishable from that of wild type littermates (Ahlgren et al. 1997) but no Isl+ epithelial cells were detected in the dorsal primordium (Fig. 24a,b). Similarly, Nkx2.2+ and glucagon+ cells were absent from the dorsal pancreatic anlage (Fig. 24c-f). In contrast, the expression of HB9 as well as that of early pancreatic markers was detected in a normal temporal and spatial manner in the dorsal pancreatic bud of Ipf1/Pdx1 -/-

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embryos (Spooner et al. 1970 and data not shown). Together, these results provide evidence that loss of Hb9 function impairs the initial stages in the dorsal pancreatic program, thus obstructing both epithelial evagination and
5   differentiation of early pancreatic cell types. They also show that the arrest in dorsal pancreatic bud development in the Hb9nlslacZ deficient mice is more severe than that observed in Ipf1/Pdx1-/- mice.

10   The signalling molecule, Sonic hedgehog (Shh), has been shown to exert an inhibitory action on pancreatic development20-22 (Apelqvist et al. 1997; Hebrok et al. 1998; Kim et al. 1997). We therefore investigated whether the impairment in initiation of dorsal pancreatic
15   development might result from the ectopic expression of Shh or Indian Hedgehog (Ihh) (Apelqvist et al. 1997; Bitgood et al. 1995) in the presumptive pancreatic epithelium. The expression of both Shh and Ihh was examined in e9.5 homozygous Hb9nlslacZ embryos. However, the domain of
20   expression of both Shh and Ihh was not expanded into the pancreatic epithelium of Hb9nlslacZ mutant embryos (Fig. 24g-j). Thus, the block in the specification of the dorsal pancreatic differentiation program in Hb9nlslacZ mutants is not due to the de-restriction of Shh or Ihh expression.

25

The ventral pancreas of Hb9-deficient mice exhibits abnormal islets that contain immature -cells.
The presence of ventral pancreatic tissue as assessed in e17.5 Hb9nlslacZ homozygous mutants_indicates that the early
30   development of the ventral pancreas is independent of Hb9 function. To analyse whether there is a late defect in the development of ventral pancreatic tissue we examined the expression of the endocrine hormones insulin (Ins) (Fig. 25a-d,g,h), glucagon (Glu) (Fig. 25a,b), somatostatin (Som)
35   (Fig. 25c,d), and the exocrine enzymes amylase (Fig. 25e,f) and carboxypeptidase A (data not shown). Each of these markers was expressed in the ventral pancreas of Hb9nlslacZ homozygous neonates and the amount and general organisation

of endocrine cells into islet-like clusters and of exocrine
cells into acinar-like structures appeared normal (Fig. 25a-
f). However, double immunohistochemical analysis revealed
a ~20% decrease in the number of Ins+-cells (Ins+/Isl1+
5   cells= ~68% in wild-types and ~53% in Hb9nlslacZ homozygous
neonates), and a ~3-fold increase in the number of Som+-
cells (Som+/Isl+ cells= ~9% in wild-types and ~25% in
Hb9nlslacZ homozygous neonates) (Fig. 25 c,d). The number
of Glu+-cells appeared unchanged in the Hb9 mutant mice
10   (Glu+/Isl1+ cells ~23% in both wild-types and Hb9nlslacZ
homozygous neonates) (Fig. 25 a,b).

We also noted a defect in the spatial organisation of
endocrine cells within individual islets. Thus, although
15   islet cell clusters were present in the ventral pancreas of
Hb9 mutants they failed to organise themselves into the
typical structure of maturing islets in which -cells are
located at the periphery and surrounds a core of -cells
(Fig. 25a-d). The Ins+-cells present in the ventral
20   pancreas of Hb9 mutant mice still expressed the
transcription factors (Edlund et al. 1998) IPF1/PDX1 (Fig.
25 g,h) and Nkx6.1 (data not shown), as well as glucokinase
(data not shown). In contrast the expression of glucose
transporter type 2 (Glut2) was virtually undetectable (Fig.
25   25 i,j). Together, these histological analyses indicate that
the ventral pancreatic epithelium generates both exocrine
and endocrine cells in the absence of Hb9 function. These
results also provide evidence that although genesis of
pancreatic ventrally derived -cells is independent of Hb9,
30   the terminal differentiation and maturation of -cells
requires Hb9 function.

Discussion.

The present study provides further insight into the
35   molecular events that control the specification of
pancreatic cell fate. Our results show that Hb9 is required
selectively for specifying the initial stages of dorsal
pancreatic development and as a consequence the dorsal

pancreatic bud does not develop in Hb9 mutant mice.   In
contrast, the ventral pancreatic epithelium develops and
generates both exocrine and endocrine cell-types, thus
revealing an early molecular distinction between the
programs of dorsal and ventral pancreatic development.
Nevertheless, the ventral pancreas of Hb9 mutant embryos
exhibits a subtle perturbation in -cell differentiation and
islet cell organisation, thus suggesting a later role for
Hb9 in the development of pancreatic -cells.

The early stages of dorsal and ventral pancreatic
development display several other distinguishing
characteristics other than the dorsal dependence on Hb9.
First, the organisation and identity of surrounding
mesenchyme and other mesodermal tissues differ at dorsal and
ventral pancreatic buds (Wessells et al. 1967; Pictet et al.
1972; Ahlgren et al. 1997; Spooner et al. 1970). Second,
pancreatic endocrine cells appear earlier in the dorsal than
in the ventral bud and third, the generation of dorsal, but
not ventral, pancreatic mesenchyme is dependent on Isl1
(Ahlgren et al. 1997). The demonstration of a selective
dorsoventral difference in the dependence on HB9 activity
thus adds to the evidence that the programs of pancreatic
differentiation dorsally and ventrally are initially
distinct. How does HB9 control the initial specification of
the dorsal pancreatic program? Our results are suggestive
of three general possibilities that relate to differences in
the timing of HB9 and IPF1/PDX1 expression dorsally and
ventrally, to the expression of a redundant activity
ventrally, and to a function of Hb9 independent of its
expression in the gut endoderm.

The first possibility that is consistent with our data
invokes a function for Hb9 in providing the dorsal gut
epithelium with the competence to respond to signals that
initiate the pancreatic differentiation program, most
critically the expression of IPF1/PDX1. In this view
Ipf1/Pdx1, once expressed, may be sufficient for the later

progression of pancreatic development, obviating the requirement for Hb9 function. The relative timing of onset of Hb9 and Ipf1/Pdx1 expression in the dorsal and ventral pancreatic buds may therefore be a critical factor in the selectivity of the mutant phenotype. Consistent with this model, our results show that IPF1/PDX1 is expressed at an earlier stage ventrally than dorsally. A second possibility is that the function of Hb9 ventrally is compensated for by a factor with a similar activity. The chick MNR2 homeobox gene is closely related to Hb9 and has been shown to have an overlapping function with the Hb9 gene in spinal cord development (Tanabe et al. 1998). Although, a mouse MNR2 gene has not yet been identified, the existence of such an activity could compensate ventrally for the loss of Hb9 function.

A third possibility is that dependence of dorsal pancreatic differentiation on Hb9 does not in fact reflect a function intrinsic to the gut epithelium. Hb9 is transiently expressed by notochord cells from ~e8 to e10. It is therefore possible ~that Hb9 acts to control dorsal pancreatic specification by regulating the expression of inductive or repressive factors secreted from the notochord. The analysis of Shh and Ihh expression in Hb9nlslacZ homozygous embryos suggests, however, that Hb9 is not involved in establishing the zone of exclusion of Shh or Ihh expression in the presumptive dorsal pancreatic region since the expression of both these genes is restricted normally in Hb9 mutant embryos. Nevertheless, the activation or repression of other genes required for pancreatic development could normally be controlled by signals from the notochord that are missing in Hb9 deficient mice. The notochord appears, however, structurally normal in the Hb9 mutant mice, the floorplate and motorneurons are generated, and both the notochord and floorplate display normal Shh expression (Arber et al. submitted for publication and data not shown). These observations suggest that many of the inducing properties of the notochord are intact in the

<u>Hb9nlslacZ</u> homozygous mice. Thus, dorsal pancreatic development seems to be dependent both on the expression of <u>Hb9</u> and on the exclusion of <u>hh</u> gene expression.

The specification of the ventral pancreatic differentiation program is independent of <u>Hb9</u> function and hence a ventral pancreas develops with both exocrine and endocrine cell types. Nevertheless, as judged from the lack of Glut2 expression in Ins+-cells present in the ventral pancreas of <u>Hb9</u> mutant mice, <u>Hb9</u> seem to be required for terminal differentiation and/or maturation of -cells. The decreased number of Ins+-cells together with the increase in the number of Som+-cells provides evidence that the establishment of these two cell-types may be coupled and that <u>Hb9</u> promotes the -cell fate on expense of Som+ -cells. Interestingly, the development of both - and -cells requires Pax4 (Sosa-Pineda et al. 1997). Thus, in the pancreas of Pax4 mutant mice there is a loss of both Ins+- and Som+-cells whereas the number of Glu+-cells increases14 (Sosa-Pineda et al. 1997). This suggests that the - and - cells originate from a common, Pax4-dependent progenitor cell and that Hb9 might function at a later stage to promote the -cell fate and characteristics. The late, ventral phenotype therefore suggests a role for <u>Hb9</u> in ensuring proper terminal differentiation of pancreatic -cells in a manner reminiscent of the role proposed for Hb9 in motor neuron differentiation (Arber et al. submitted for publication).

Genetic studies in mice have shown that several transcription factors have important roles for pancreatic development and/or glucose homeostasis (Slack et al. 1995). Many of these genes have been linked to human diabetic or pancreatic syndromes, thus demonstrating a conservation in the function of genes that control pancreatic development in mice and humans (Edlund et al. 1998) In humans as in mice, homozygosity for mutations in the human <u>Ipf1</u>gene results in complete pancreas agenesis (Ahlgren et al. 1996; Jonsson et

al. 1994; Offield et al. 1996; Stoffers et al. 1997).
Maturity Onset Diabetes of the Young (MODY) 4 has been
linked to heterozygosity for mutations in the human <u>Ipf1</u>
gene (Stoffers et al. 1997) and similarly, mice
heterozygous for deletions in the <u>Ipf1/Pdx1</u> gene have
impaired glucose tolerance (Ahlgren et al. 1998; Dutta et
al. 1998). The human <u>Hb9</u> gene, <u>HLXB9</u>, has been linked to
dominant inherited forms of sacral agenesis. (Ross et al.
1998). No pancreatic or diabetic complications have yet
been reported in these patients but spontaneous forms of
this syndrome are known to occur in infants of type1
diabetic mothers (Ross et al. 1998; Kalter et al. 1993).
These observations raise the possibility that expression or
function of <u>Hb9</u> might be sensitive to elevated glucose
levels in a manner similar to that reported for <u>Ipf1/Pdx1</u>
(Sharma et al. 1995; Matsuoka et al. 1997; Olson et al.
1998). The apparent immature status of the -cells in the
ventral pancreas of <u>Hb9</u> mutant mice also suggests that
impaired <u>Hb9</u> function, or expression, may turn out to be
associated with development of diabetes. Whether mutations
in <u>HLXB9</u> are also associated with human dorsal pancreatic
agenesis syndromes (Wildling et al. 1993) remains to be
determined.

METHODS:
Animals: The generation of <u>Hb9nlslacZ</u> mice has been
described elsewhere (Arber et al. submitted for publication)
and mutant mice were obtained from our local breeding
colony. Mice and embryos were genotyped as described
elsewhere (Arber et al. submitted for publication).

In situ hybridization and immunohistochemistry.
<u>In situ</u> hybridization using a rat <u>Shh</u> (Arber et al.
submitted for publication) and a mouse <u>Ihh</u> probe, (Kim et
al. 1997) kindly provided by A.P. McMahon, was carried out
as described (Ahlgren et al. 1996). Immunohistochemistry
was performed as previously described (Ahlgren et al. 1996).
The following primary antibodies were used and diluted as

indicated: guinea pig anti-glucagon (Linco) 1:1000, guinea
pig anti-insulin (DAKO) 1:10000, mouse anti-HB9, (Tanabe et
al. 1998) rabbit anti-HB9 (Arber et al. submitted for
publication) , 1:10 and 1:200 respectively, rabbit anti-
5   human -amylase (Sigma) 1:4000, rabbit anti-Nkx2.2 (Briscoe
et al. manuscript in preparation), 1:2000, rabbit anti-Isl1
(Ahlgren et al. 1997), 1:250, rabbit anti-IPF1/PDX-1
(Ohlsson et al. 1993), 1:400, rat anti-somatostatin
antibodies (Bender MedSystems) 1:1000, rabbit anti Glut2
10   (Ahlgren et al. 1998) (Inst. of Pharm. & Tox., Lausanne,
Switzerland), 1:200. When mou e anti-HB9 antibody was used,
the blocking step was performed using a Mouse to Mouse kit
(ScyTek). When double staining was carried out, a second
blocking step using swine anti-rabbit IgG (DAKO) diluted
15   1:20 was included. The secondary antibodies used were
diluted as follows: biotinylated anti-mouse (Jackson)
1:200, biotinylated anti-rat (Vector) 1:200, fluorescein
anti-guinea pig (Jackson) 1:200, and Cy3 anti-rabbit
(Jackson) 1:200. Streptavidin-FITC (Jackson) 1:200 was
20   applied to detect biotinylated secondary antibodies. The
numbers of Ins-, Glu- and Som-positive cells (n=800-1500
cells from 3 different wild-type and mutant mice
respectively) were counted using anti Isl-1 antibodies
(Ahlgren et al. 1997) as a marker for endocrine cells.
25

Whole-mount X-gal staining.
Fixed Hb9nlslacZ heterozygous and homozygous embryos were
washed in 0.1M PB (pH7.4), 2mM MgCl2, 5mM EGTA,
permeabilized in 0.1M PB (pH7.4), 2mM MgCl2, 0.01% sodium
30   desoxycholate, 0.02% NP40 and X-gal (Saveen) stained for 3
hours or overnight at 37C. Embryos were post-fixed in 4%
paraformaldehyde in PBS (pH7.4) at 4C for 30 minutes before
being dehydrated successively from PBS (pH7.4) to 100%
methanol and cleared in 1:2 benzyl alcohol:benzyl benzoate
35   for photography. Later they were rehydrated and incubated
overnight at 4C in 30% sucrose in PBS (pH7.4) before being
embedded in Tissue-Tek (Sakura) for cryostat sectioning.

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gacgcgctgc tggctgtcga tccccccaag gcggcggcgc agagcgctcc gctggccctg 120
gtcaccggcg gctccggcgg cggcagccct ccgtcttcgt cgtcctcctc gtcgtcgtcg 180

```
tcctcctctt cttccgagct ccccgccgac tgcccgcgca ccgacagccc ctctccgcct 240
cgcctgctgc ccgcgcactg cgcgctgctg cccaaagccg ccttcctggg cgggggggga 300
cccggggggcg gccacccgca gcaccacgcc ctggggctgc accccgcggg gccgggcggg 360
ccgggcctct acgggcaccc ggtgtacggc tacccggcgt tgggcgggca gcacccggcg 420
ctctcctatt cctattcgca agtgcaggga gcgcaccccg cgcatccctc cgccgacccc 480
atcaagctga gcgccggcac ctttcagctg gaccagtggc tgcgggcgag cacggccggc 540
atgatcctgc ccaaaatgcc cgacttcggc tctcaggcgc agtccaacct gctggggaag 600
tgccggcggc cgcgcaccgc cttcaccagc cagcagctgc tggagctgga gcaccagttc 660
aaactcaaca agtacctctc ccggcccaag cgcttcgagg tggccacgtc gctgatgctc 720
accgagacgc aggtgaagat ttggttccag aaccgccgca tgaaatggaa gcgccagaaa 780
aaggcgaagg agcaggcggc gcaggaggca gagaacgaga aaggaggagg aggaggagag 840
gacaaaagcg ggccgaggga actgctgctg cccggcccgg agaaaggcgg cgggaggcgg 900
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tgagcccacg gccgccccgt cggggccgcc cccggcaacg gagcctcctg gccccgctct 1140
ccatcccgct ctcccatccc tccctgctcg gagggggacg cggaaaggga tctcccgtct 1200
gccgagcggg agggaggatt cacacagtgt tattattgac tgagaagcgg ccacgacttg 1260
agccccctc cccgccccgc cctatcggaa ccgtttcctt cttaccatat atcgggaaaa 1320
gtgtttatgt catgaacgtt aaaactgctg cagatctcaa tactgtcttt attttgtata 1380
tcctatttat aaaaaaggca aaatgaattc ctctacttat gcatgctaaa ttattaccca 1440
gccccttccg cctgaggtgg gggggaggaa tataaataaa gagcgttttg tactgtgaaa 1500
aaaaaaaaaa aaaa                                                  1514
```

What is claimed is:

1.   An isolated nucleic acid molecule encoding a motor neuron restricted pattern, MNR2, protein.

2.   The isolated nucleic acid of claim 1 which is a DNA molecule.

3.   The isolated nucleic a id of claim 2 which is a cDNA molecule.

4.   The isolated nucleic acid of claim 1 which is a RNA molecule.

5.   The isolated nucleic acid of claim 1 operatively linked to a promoter of RNA transcription.

6.   A vector which comprises the isolated nucleic acid of claim 1, operatively linked to a promoter of RNA transcription.

7.   A plasmid comprising the vector of claim 6.

8.   The plasmid of claim 7 designated pMNR2 (ATCC Accession No. 203294.

9.   The isolated nucleic acid molecule of claim 3, wherein the nucleic acid molecule encodes a chick MNR2 protein.

10.   The isolated nucleic acid molecule of claim 9, wherein the nucleic acid molecule encodes a MNR2 protein comprising the amino acid sequence set forth in SEQ ID NO: 1.

11. The isolated nucleic acid molecule of claim 3, wherein the nucleic acid molecule encodes a mammalian MNR2 protein.

5   12. The isolated nucleic acid molecule of claim 11, wherein the mammalian MNR2 protein is mouse, rat, or human protein.

13. The isolated nucleic acid of claim 3 which comprises the nucleic acid sequence set forth in SEQ ID NO: 1.

10

14. A host cell containing the vector of claim 6.

15. The host cell of claim 14, wherein the cell is selected from a group consisting of a bacterial cell, a plant cell, an insect cell, and a mammalian cell.

15

16. A method of producing a polypeptide having the biological activity of a mammalian MNR2 which comprises growing the host cells of claim 15 under suitable conditions permitting production of the polypeptide.

20

17. The method of claim 16 further comprising recovering the produced polypeptide.

25

18. An isolated nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the nucleic acid molecule of claim 1.

30

19. The isolated nucleic acid of claim 18 which is a DNA molecule.

35   20. The isolated nucleic acid of claim 18 which is a RNA molecule.

21.   An isolated nucleic acid molecule capable of
specifically hybridizing with a unique sequence
included within the sequence of a nucleic acid
molecule which is complementary to the nucleic acid
molecule of claim 1.

22.   The isolated nucleic acid of claim 21 which is a DNA
molecule.

23.   The isolated nucleic acid of claim 21 which is a RNA
molecule.

24.   An antisense oligonucleotide having a nucleic acid
sequence capable of specifically hybridizing to an
mRNA molecule encoding a MNR2 protein.

25.   An antisense oligonucleotide having a nucleic acid
sequence capable of specifically hybridizing to the
cDNA molecule of claim 3.

26.   An antisense oligonucleotide having a nucleic acid
sequence capable of specifically hybridizing to the
RNA molecule of claim 4.

27.   A purified MNR2 protein.

28.   A purified MNR2 protein encoded by the isolated
nucleic acid of claim 1.

29.   A purified unique polypeptide fragment of the MNR2
protein of claim 28.

30.   The MNR2 protein of claim 28 having substantially the
same amino acid sequence as set forth in SEQ ID NO: 1.

31.   The MNR2 protein of claim 28 having the amino acid
sequence as set forth in SEQ ID NO: 1.

32. The MNR2 protein of claim 31 which is a vertebrate MNR2 protein.

33. The vertebrate MNR2 protein of claim 32 which is a chick, mouse, rat, or human MNR2 protein.

34. A monoclonal antibody directed to an epitope of an MNR2 protein of claim 33.

35. A monoclonal antibody of claim 34 directed to a chick, mouse, rat or human MNR2 protein.

36. A polyclonal antibody directed to an epitope of the MNR2 protein of claim 31.

37. A polyclonal antibody of claim 36 directed to a chick, mouse, rat or human MNR2 protein.

38. A method of inducing differentiation somatic motor neurons which comprises expressing MNR2 protein in neural progenitor cells.

39. The method of claim 38, wherein the expression of MNR2 protein induces expression of transcription factors Isl2, Lim 3 and HB9.

40. The method of claim 38, wherein the neural progenitor cells are spinal cord motor neuron progenitor cells or hindbrain motor neuron progenitor cells.

41. A transgenic nonhuman mammal which comprises an isolated DNA molecule of claim 2.

42. The transgenic nonhuman mammal of claim 41, wherein the DNA encoding a MNR2 protein is operatively linked to tissue specific regulatory elements.

43.   A method of determining physiological effects of expressing varying levels of MNR2 protein in a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman animals, each transgenic nonhuman mammal expressing a different amount of MNR2 protein.

44.   A method of producing the isolated protein of either of claims 27 or 28 which comprises:

      (a)   inserting a nucleic acid molecule encoding a MNR2 protein into a suitable vector;

      (b)   introducing the resulting vector into a suitable host cell;

      (c)   selecting the introduced host cell for the expression of the MNR2 protein;

      (d)   culturing the selected cell to produce the MNR2 protein; and

      (e)   recovering the MNR2 protein produced.

45.   A method of inducing differentiation of somatic motor neurons in a subject comprising administering to the subject the purified MNR2 protein of either of claims 27 or 28 in an amount effective to induce differentiation of somatic motor neurons in the subject.

46.   The method of claim 45 wherein the subject is a mammal.

47.   The method of claim 46, wherein the mammal is a mouse, rat or human.

48. A pharmaceutical composition comprising a purified MNR2 protein of either of claims 27 or 28 and a pharmaceutically acceptable carrier.

5 49. A method for treating a subject afflicted with an abnormality associated with a lack of one or more normally functioning motor neurons which comprises introducing an amount of the pharmaceutical composition of claim 48 effective to generate somatic

10 motor neurons from undifferentiated motor neuron precursor cells in the subject, thereby treating the subject afflicted with the abnormality associated with the lack of one or more normally functioning motor neurons.

15
50. A method of treating a subject afflicted with a neurodegenerative disease which comprises introducing an amount of the pharmaceutical composition of claim 48 effective to generate somatic motor neurons from undifferentiated precursor motor neuron cells in the

20 subject, thereby treating the subject afflicted with the neurodegenerative disease.

51. The method of claim 49 wherein the generation of motor neurons from undifferentiated precursor motor neuron

25 cells alleviates a chronic neurodegenerative disease.

52. A method of treating a subject afflicted with an acute nervous system injury which comprises introducing an amount of pharmaceutical composition of claim 48 effective to generate motor neurons from

30 undifferentiated precursor motor neuron cells in a subject, thereby treating the subject afflicted with the acute nervous system injury.

35
53. The method of claim 52 wherein the acute nervous system injury is localized to a specific central axon which comprises surgical implantation of the

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pharmaceutical composition of claim 48 effective to generate motor neurons from undifferentiated precursor motor neuron cells located proximal to the specific central axon, so as to alleviate the acute nervous system injury localized to a specific central axon, thereby treating the subject afflicted with the acute nervous system injury.

54. The method of claim 51 wherein the chronic neurodegenerative disease is spinal muscular atrophies.

55. The method of claim 51 wherein the chronic neurodegenerative disease is myotrophic lateral sclerosis.

56. A method for diagnosing a chronic neurodegenerative disease associated with the expression of a MNR2 protein in a sample from a subject which comprises:

(a) obtaining DNA from the sample of the subject suffering from the chronic neurodegenerative disease;

(b) performing a restriction digest of the DNA with a panel of restriction enzymes;

(c) separating the resulting DNA fragments by size fractionation;

(d) contacting the resulting DNA fragments with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a MNR2 protein, wherein the sequence of a nucleic acid molecule encoding a MNR2 protein is linked at a specific break point to a

specified nucleic acid sequence and labeled with
a detectable marker;

(e)   detecting labeled bands which have hybridized to
the nucleic acid probe capable of specifically
hybridizing with a unique sequence included
within the sequence of a nucleic acid molecule
encoding a MNR2 protein, wherein the sequence of
a nucleic acid molecule encoding a MNR2 protein
is linked at a specific break point to a
specified nucleic acid sequence to create a
unique band pattern specific to the DNA of
subjects suffering from the chronic
neurodegenerative disease;

(f)   preparing DNA obtained from a sample of a subject
for diagnosis by steps (a-e); and

(g)   comparing the detected band pattern specific to
the DNA obtained from a sample of subjects
suffering from the chronic neurodegenerative
disease from step (e) and the DNA obtained from
a sample of the subject for diagnosis from step
(f) to determine whether the patterns are the
same or different and to diagnose thereby
predisposition to the chronic neurodegenerative
disease if the patterns are the same.

57.   A method for diagnosing a chronic neurodegenerative
disease associated with the expression of a MNR2
protein in a sample from a subject which comprises:

(a)   obtaining RNA from the sample of the subject
suffering from chronic neurodegenerative disease;

(b)   separating the RNA sample by size fractionation;

(c)   contacting the resulting RNA species with a
      nucleic acid probe capable of specifically
      hybridizing with a unique sequence included
      within the sequence of a nucleic acid molecule
      encoding a MNR2 protein, wherein the sequence of
      a nucleic acid molecule encoding a MNR2 protein
      is linked at a specific break point to a
      specified nucleic acid sequence and labeled with
      a detectable marker;

(d)   detecting labeled bands which have hybridized to
      the RNA species to create a unique band pattern
      specific to the RNA of subjects suffering from
      the chronic neurodegenerative disease;

(e)   preparing RNA obtained from a sample of a subject
      for diagnosis by steps (a-d); and

(f)   comparing the detected band pattern specific to
      the RNA obtained from a sample of subjects
      suffering from the chronic neurodegenerative
      disease from step (d) and the RNA obtained from
      a sample of the subject for diagnosis from step
      (f) to determine whether the patterns are the
      same or different and to diagnose thereby
      predisposition to the chronic neurodegenerative
      disease if the patterns are the same.

58.   A functionally equivalent analog of MNR2 that induces
      MNR2 differentiation of neural progenitor cells.

59.   A functionally equivalent analog of MNR2 that prevents
      MNR2 differentiation of neural progenitor cells.

60.   A method of treating a subject afflicted with a
      neuromuscular disease which comprises introducing an
      amount of the pharmaceutical composition of claim 48

effective to activate acetylcholine to activate muscle cells.

61.   An isolated nucleic acid molecule encoding a homeobox, HB9, protein.

62.   The isolated nucleic acid of claim 61 which is a DNA molecule.

63.   The isolated nucleic acid of claim 62 which is a cDNA molecule.

64.   The isolated nucleic acid of claim 61 which is a RNA molecule.

65.   The isolated nucleic acid of claim 61 operatively linked to a promoter of RNA transcription.

66.   A vector which comprises the isolated nucleic acid of claim 61, operatively linked to a promoter of RNA transcription.

67.   A plasmid comprising the vector of claim 66.

68.   The plasmid of claim 67 designated pHB9

69.   The isolated nucleic acid molecule of claim 63, wherein the nucleic acid molecule encodes a chick HB9 protein.

70.   The isolated nucleic acid molecule of claim 69, wherein the nucleic acid molecule encodes a HB9 protein comprising the amino acid sequence set forth in SEQ ID NO: 1.

71.   The isolated nucleic acid molecule of claim 63, wherein the nucleic acid molecule encodes a mammalian HB9 protein.

72. The isolated nucleic acid molecule of claim 71, wherein the mammalian HB9 protein is mouse, rat, or human protein.

73. The isolated nucleic acid of claim 63 which comprises the nucleic acid sequence set forth in SEQ ID NO: 1.

74. A host cell containing the vector of claim 66.

75. The host cell of claim 64, wherein the cell is selected from a group consisting of a bacterial cell, a plant cell, an insect cell, and a mammalian cell.

76. A method of producing a polypeptide having the biological activity of a mammalian HB9 which comprises growing the host cells of claim 65 under suitable conditions permitting production of the polypeptide.

77. The method of claim 66 further comprising recovering the produced polypeptide.

78. An isolated nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the nucleic acid molecule of claim 61.

79. The isolated nucleic acid of claim 68 which is a DNA molecule.

80. The isolated nucleic acid of claim 68 which is a RNA molecule.

81. An isolated nucleic acid molecule capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule which is complementary to the nucleic acid molecule of claim 61.

82.   The isolated nucleic acid of claim 81 which is a DNA molecule.

5   83.   The isolated nucleic acid of claim 81 which is a RNA molecule.

      84.   An antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to an mRNA molecule encoding a HB9 protein.

10

      85.   An antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to the cDNA molecule of claim 63.

15

      86.   An antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to the RNA molecule of claim 64.

20   87.   A purified HB9 protein.

      88.   A purified HB9 protein encoded by the isolated nucleic acid of claim 61.

25   89.   A purified unique polypeptide fragment of the HB9 protein of claim 88.

      90.   The HB9 protein of claim 88 having substantially the same amino acid sequence as set forth in SEQ ID NO: 1.

30

      91.   The HB9 protein of claim 88 having the amino acid sequence as set forth in SEQ ID NO: 1.

      92.   The HB9 protein of claim 91 which is a vertebrate HB9

35   protein.

      93.   The vertebrate HB9 protein of claim 92 which is a chick, mouse, rat, or human HB9 protein.

94. A monoclonal antibody directed to an epitope of an HB9 protein of claim 93.

95. A monoclonal antibody of claim 94 directed to a chick, mouse, rat or human HB9 protein.

96. A polyclonal antibody directed to an epitope of the HB9 protein of claim 91.

97. A polyclonal antibody of claim 96 directed to a chick, mouse, rat or human HB9 protein.

98. A method of inducing differentiation somatic motor neurons which comprises expressing HB9 protein in neural progenitor cells.

99. The method of claim 98, wherein the expression of HB9 protein induces expression of transcription factors Isl2, Lim 3 and HB9.

100. The method of claim 98, wherein the neural progenitor cells are spinal cord motor neuron progenitor cells or hindbrain motor neuron progenitor cells.

101. A transgenic nonhuman mammal which comprises an isolated DNA molecule of claim 62.

102. The transgenic nonhuman mammal of claim 101, wherein the DNA encoding a HB9 protein is operatively linked to tissue specific regulatory elements.

103. A method of determining physiological effects of expressing varying levels of HB9 protein in a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman animals, each transgenic nonhuman mammal expressing a different amount of HB9 protein.

104. A method of producing the isolated protein of either of claims 87 or 88 which comprises:

    (a) inserting a nucleic acid molecule encoding a HB9 protein into a suitable vector;

    (b) introducing the resulting vector into a suitable host cell;

    (c) selecting the introduced host cell for the expression of the HB9 protein;

    (d) culturing the selected cell to produce the HB9 protein; and

    (e) recovering the HB9 protein produced.

105. A method of inducing differentiation of somatic motor neurons in a subject comprising administering to the subject the purified HB9 protein of either of claims 87 or 88 in an amount effective to induce differentiation of somatic motor neurons in the subject.

106. The method of claim 105 wherein the subject is a mammal.

107. The method of claim 106, wherein the mammal is a mouse, rat or human.

108. A pharmaceutical composition comprising a purified HB9 protein of either of claims 87 or 88 and a pharmaceutically acceptable carrier.

109. A method for treating a subject afflicted with an abnormality associated with a lack of one or more normally functioning motor neurons which comprises

introducing an amount of the pharmaceutical composition of claim 108 effective to generate somatic motor neurons from undifferentiated motor neuron precursor cells in the subject, thereby treating the subject afflicted with the abnormality associated with the lack of one or more normally functioning motor neurons.

110. A method of treating a subject afflicted with a neurodegenerative disease which comprises introducing an amo·nt of the pharmaceutical composition of claim 108 effective to generate somatic motor neurons from undifferentiated precursor motor neuron cells in the subject, thereby treating the subject afflicted with the neurodegenerative disease.

111. The method of claim 109 wherein the generation of motor neurons from undifferentiated precursor motor neuron cells alleviates a chronic neurodegenerative disease.

112. A method of treating a subject afflicted with an acute nervous system injury which comprises introducing an amount of pharmaceutical composition of claim 108 effective to generate motor neurons from undifferentiated precursor motor neuron cells in a subject, thereby treating the subject afflicted with the acute nervous system injury.

113. The method of claim 112 wherein the acute nervous system injury is localized to a specific central axon which comprises surgical implantation of the pharmaceutical composition of claim 108 effective to generate motor neurons from undifferentiated precursor motor neuron cells located proximal to the specific central axon, so as to alleviate the acute nervous system injury localized to a specific central axon,

thereby treating the subject afflicted with the acute
nervous system injury.

114. The method of claim 111 wherein the chronic
neurodegenerative disease is spinal muscular
atrophies.

115. The method of claim 111 wherein the chronic
neurodegenerative disease is myotrophic lateral
sclerosis.

116. A method for diagnosing a chronic neurodegenerative
disease associated with the expression of a HB9
protein in a sample from a subject which comprises:

(a) obtaining DNA from the sample of the subject
suffering from the chronic neurodegenerative
disease;

(b) performing a restriction digest of the DNA with
a panel of restriction enzymes;

(c) separating the resulting DNA fragments by size
fractionation;

(d) contacting the resulting DNA fragments with a
nucleic acid probe capable of specifically
hybridizing with a unique sequence included
within the sequence of a nucleic acid molecule
encoding a HB9 protein, wherein the sequence of
a nucleic acid molecule encoding a HB9 protein is
linked at a specific break point to a specified
nucleic acid sequence and labeled with a
detectable marker;

(e) detecting labeled bands which have hybridized to
the nucleic acid probe capable of specifically
hybridizing with a unique sequence included

within the sequence of a nucleic acid molecule
encoding a HB9 protein, wherein the sequence of
a nucleic acid molecule encoding a HB9 protein is
linked at a specific break point to a specified
nucleic acid sequence to create a unique band
pattern specific to the DNA of subjects suffering
from the chronic neurodegenerative disease;

(f)   preparing DNA obtained from a sample of a subject
        for diagnosis by steps (a-e); and

(g)   comparing the detected band pattern specific to
        the DNA obtained from a sample of subjects
        suffering from the chronic neurodegenerative
        disease from step (e) and the DNA obtained from
        a sample of the subject for diagnosis from step
        (f) to determine whether the patterns are the
        same or different and to diagnose thereby
        predisposition to the chronic neurodegenerative
        disease if the patterns are the same.

117.   A method for diagnosing a chronic neurodegenerative
        disease associated with the expression of a HB9
        protein in a sample from a subject which comprises:

(a)   obtaining RNA from the sample of the subject
        suffering from chronic neurodegenerative disease;

(b)   separating the RNA sample by size fractionation;

(c)   contacting the resulting RNA species with a
        nucleic acid probe capable of specifically
        hybridizing with a unique sequence included
        within the sequence of a nucleic acid molecule
        encoding a HB9 protein, wherein the sequence of
        a nucleic acid molecule encoding a HB9 protein is
        linked at a specific break point to a specified

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nucleic acid sequence and labeled with a detectable marker;

(d) detecting labeled bands which have hybridized to the RNA species to create a unique band pattern specific to the RNA of subjects suffering from the chronic neurodegenerative disease;

(e) preparing RNA obtained from a sample of a subject for diagnosis by steps (a-d); and

(f) comparing the detected band pattern specific to the RNA obtained from a sample of subjects suffering from the chronic neurodegenerative disease from step (d) and the RNA obtained from a sample of the subject for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the chronic neurodegenerative disease if the patterns are the same.

118. A functionally equivalent analog of HB9 that induces HB9 differentiation of neural progenitor cells.

119. A functionally equivalent analog of HB9 that prevents HB9 differentiation of neural progenitor cells.

120. A method of treating a subject afflicted with a neuromuscular disease which comprises introducing an amount of the pharmaceutical composition of claim 108 effective to activate acetylcholine to activate muscle cells.

121. A method of treating an embryo afflicted with sacral agenesis which comprises introducing the isolated nucleic acid of claim 61 into the embryo.

122. A method of treating an embryo afflicted with sacral agenesis which comprises introducing an amount of the pharmaceutical composition of claim 108.

5   123. A method of treating an embryo lacking HB9 expression which comprises introducing the isolated nucleic acid of claim 61.

     124. A method of treating an embryo lacking HB9 expression
10   which comprises introducing an amount of the pharmaceutical composition of claim 108.

FIG. 1A Single cell cDNA identification

FIG. 1B

FIG. 1C   FIG. 1D   FIG. 1E

Stage 12   Stage 16   Stage 20

FIG. 1F   FIG. 1G   FIG. 1H
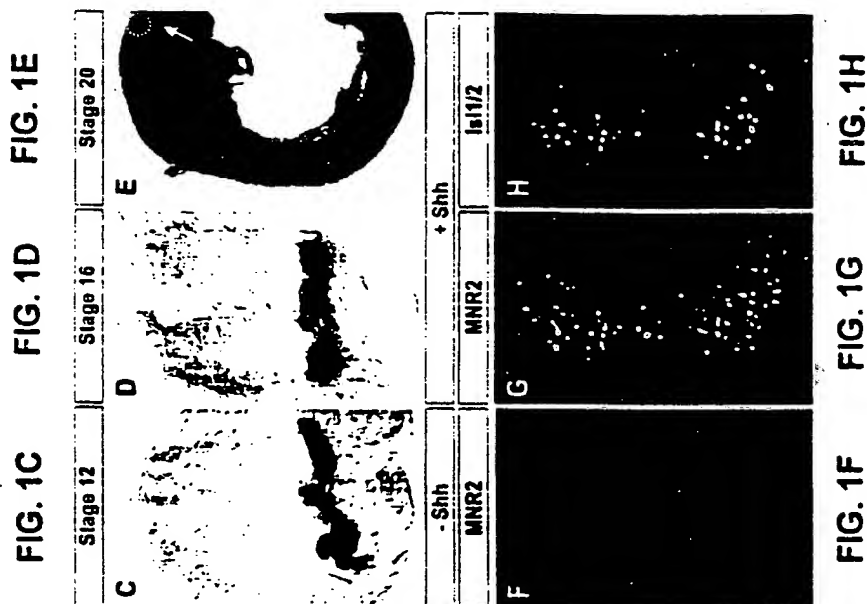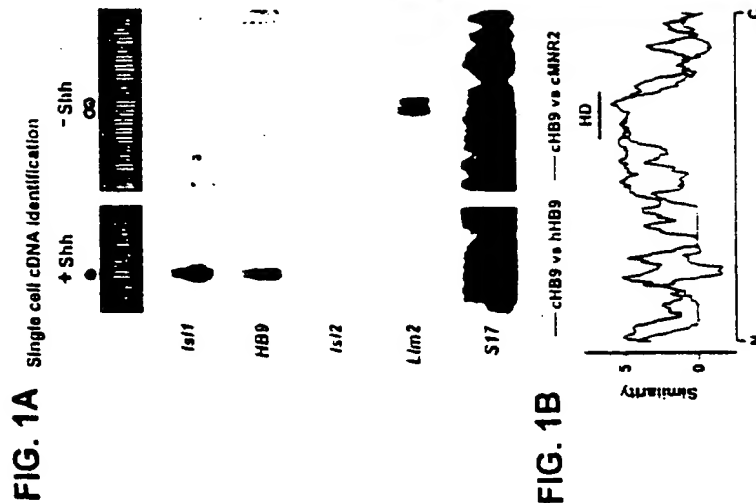
FIG. 2A   FIG. 2B   FIG. 2C   FIG. 2D   FIG. 2E   FIG. 2F
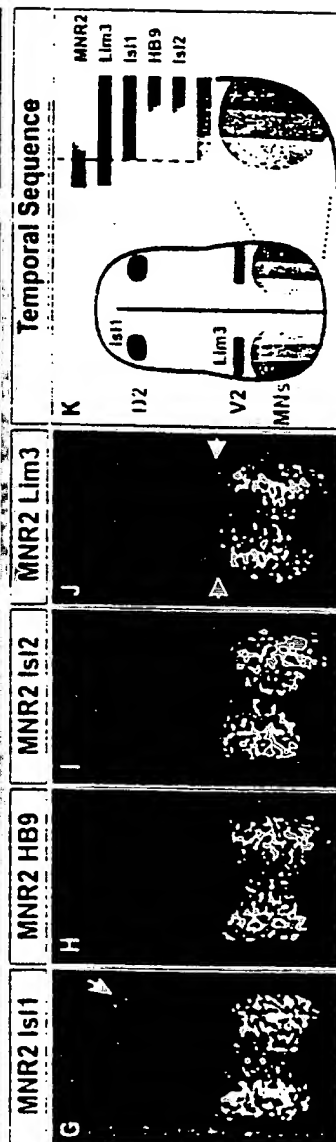
FIG. 2G   FIG. 2H   FIG. 2I   FIG. 2J   FIG. 2K

FIG. 3E · FIG. 3D · FIG. 3C · FIG. 3B · FIG. 3A · FIG. 3J · FIG. 3I · FIG. 3H · FIG. 3G · FIG. 3F

FIG. 4A FIG. 4B FIG. 4C FIG. 4D

MNR2 Cyn1 | MNR2 cells | Isl1/2 Cyn1 | Ectopic Isl1

RCAS(B) MNR2

ectopic HD | Lim3 BrdU | Isl1 Lim3 | MNR2 neurons

FIG. 4E FIG. 4F FIG. 4G FIG. 4H

RCAS(B)MNR2 3'UTRΔ

5' coding probe

FIG. 4I

3' UTR probe

FIG. 4J
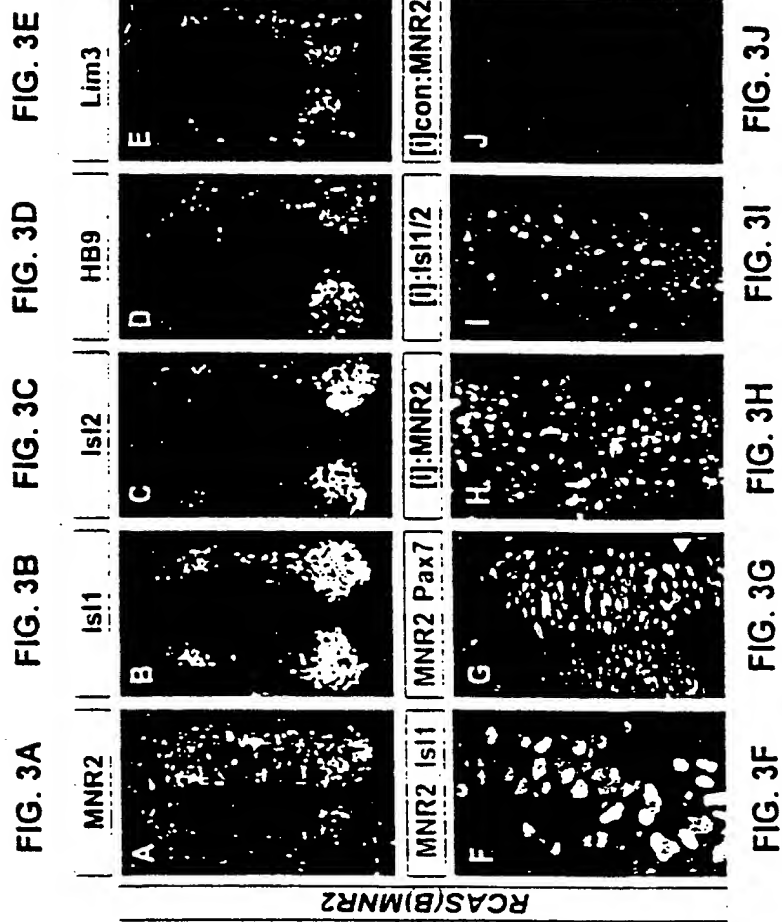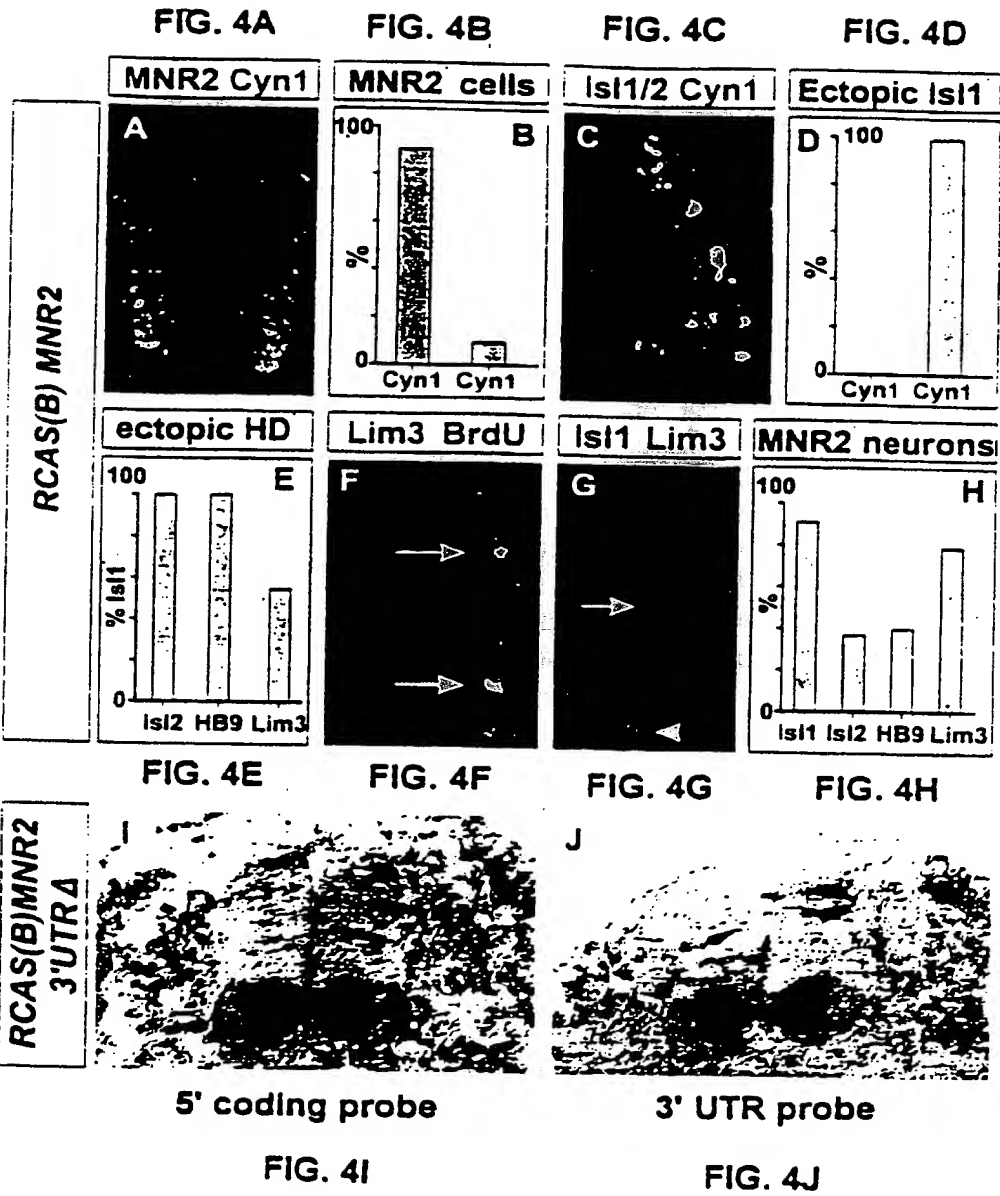
FIG. 5A　FIG. 5B　FIG. 5C　FIG. 5D　FIG. 5E
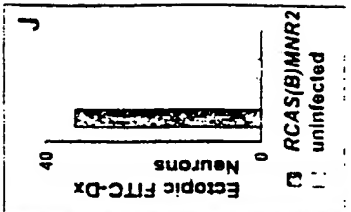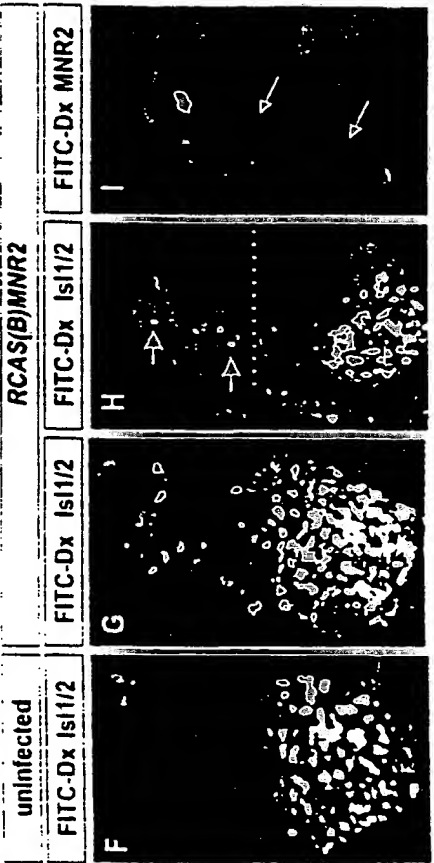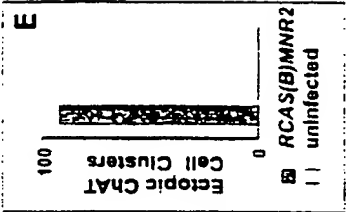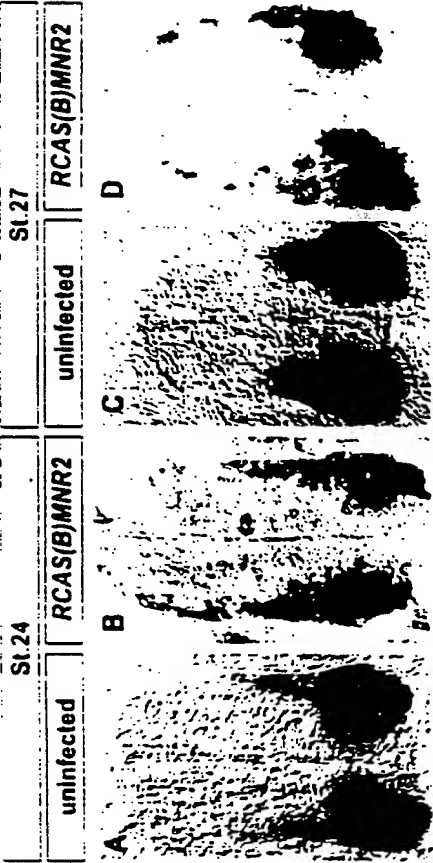
FIG. 5F　FIG. 5G　FIG. 5H　FIG. 5I　FIG. 5J

FIG. 6A Isl1
FIG. 6B MNR2
FIG. 6C Lim3
FIG. 6D Isl2
FIG. 6E HB9
FIG. 6F Isl1
FIG. 6G Isl2
FIG. 6H Isl1 Isl2
FIG. 6I HB9
FIG. 6J Isl1 HB9
FIG. 6K MNR2 Isl 1
FIG. 6L Isl 2
FIG. 6M HB9
FIG. 6N MNR2, Isl 1 cells

RCAS(A)Isl1
RCAS(B)MNR2
RCAS(B)MNR2 RCAS(A)Isl1

FIG. 6O

FIG. 6P-1   FIG. 6P-2   FIG. 6P-3

FIG. 6P-4   FIG. 6P-5   FIG. 6P-6

FIG. 6P-7   FIG. 6P-8   FIG. 6P-9

FIG. 7A   FIG. 7B   FIG. 7C   FIG. 7D   FIG. 7E   FIG. 7F   FIG. 7G

FIG. 7H   FIG. 7I   FIG. 7J   FIG. 7K   FIG. 7L   FIG. 7M   FIG. 7N

FIG. 8

FIG. 9A

Interneuron Pattern

D2

V2

FIG. 9B

P.CAS S.MNR2

FIG. 9C

LH2

FIG. 9D

Isl1

FIG. 9E

LH2   Isl1

D1

Brn3.0

Isl1

Brn3.0   Isl1

D2

FIG. 9F

FIG. 9G

FIG. 9H

FIG. 9I   FIG. 9J   FIG. 9K

| En1 | Isl1 | En1   Isl1 |

FIG. 9L   FIG. 9M   FIG. 9N

FIG. 10

SEQ. ID NO. 1

cMNR2-protein

```
  1 MIKPMEKSQN FRIEALLAEK PPRSASPPGL SPAGSPGPAG RTDTPSPRAP QAATPIGPAG
 61 FVPKPGLLHL PGPGLGTLPA LYPPAVYPLP ALGGQHAAFA YTAFPQLPPP GAEHLKAAVA
121 GSFPIEQWIR AGMLVPRLSD FHATPQSALM GKSRRPRTAF TSQLLELEN QFKLNKYLSR
181 PKRFEVATSL MLTETQVKIW FQNRRMKWKR SRKAKEQGMA VEPEKPRGLG KADESLLPSQ
241 PQGQAGDSPE FVGCSPGTGF LCRSAELGYD PDSSCSGGEE DEEEDDGMD TAERKMGSVL
```

FIG. 11   cMNR2-dna

SEQ. ID NO. 2

```
   1 CAGATCTGCT CCCAGATGCT CTGCCTCTCC TCGAAGGCCA GAGTCGGTGG GTCCGGGCCA
  61 GCTCTGCTCC TGCTCACCCG CCTGTCCCAG AGCAGCCAAG GCTTTCATCT CCACCTGTTT
 121 CTGGTGCCTT CACCTGGAGA AGACCAAACC GAGCAAATAA ATAACAATCT GCCCGTATGC
 181 ACCTGCTCCA TGGCTTCTT GGGCGGATAG ACGATGCACG GTTGTGGCCC CTGCGTGCAG
 241 CCAGCTCGGG CCCGCTGATG TCCCCGTGCC AAAGAGGTGC AAAGAGGAAC GGCACGGGGT
 301 GTGAAGAGCA GGATCGGGCC CCGGGGTGTG GCGGAAGCCG CGGGAGGCAG CAGGTGGAGG
 361 GCCGGGCCGA CGGGGCGGGG GGCCGGCGGG GAGCCAATAG GGAGCTGGGG AGGAGCGGTG
 421 GGGGGGTTAA AACCCCCCCG GTGGCGGCGG GCAAGCGAGT GCCCGGGAGG AGGAGCGGTG
 481 AGGAGGGCTG CCCCTGAGGG CAGCGGAGGC CGGCGGCGCC ATGCCCGGCG ATGCCCGGCGG
 541 GTGCCGGGGT GGCCCGGGGC TGCCCGGCCG GCGGCCATGC ACAAACCCAT GGAGAAGTCC
 601 GAGGCACGCT GCGTTTGCGC GGGGCCCGGC GCTCCTGGCT GAGAAGCCGC CTCTCCTCCG
 661 CAAAACTTCC GCATCGAGGC CCCGGGGCAG CCGGCGCGTA CCGACACCCC CTCGCCTCGG
 721 GGGCTCAGCC CCGGCGGGCAG CCCGCCACCC TCCCCAAACC TCCCCAAACC CGGCTTGCTG
 781 GCTCCCCAGG CCGGCCACCCC CCTCGGCCCG CCGGCCCTCT ACCCGCCTGC CGTGTACCCG
 841 CACCTCCCCG GCCCCGGGCT GGGCACCCTG CCGGCCCTCT TTCGCCTACA CCAGCTGCCG
 901 CTGCCGGCCT TGGGGGGCCA GCACGCCGCT GTGGCCGGTT CCTTCCCGCT GGAGCAGTCG
 961 CCGCCCCGGCG CCCAGCACCT GAAGGCGGCG GCCGAGGCTC TCCGACTTCC ACAGTCCCGCC
1021 ATCCGAGCCG GGATGCTCGT GCCCCGCACCG GCCCCGCACC GCCTTCACCA GCTGAGCTTG
1081 TTGATGGGAA AGTCGGCGCG CAAGTATCTG GCCTTCACCA AGCGCTTCGA GGTGGCCACG
1141 GAGAACCAGT TCAAGCTCAA TCACTGAGAC TCCAGCGCCA ATCTGGTTCC AGAACCCCG CATGAAGTGG
1201 TCGCTGATGC TCACTGAGAC GCAGGTGAAG ATGGCAGTGG AGCCCGAGAA GCCACGGCGG
1261 AAGCGGAGCC GCAAAGCCAA GGAGCAGGGG TCTGCTGCCC AGGGACAGGC TGGTGACAGC
1321 CTTGGCAAAG CTGATGAGAG CCCCGGAACG GCCTCCTGT GCCGCAGCGC GCAGCTGGGC
1381 CCCGAGTTTG TGGGGTGGCAG CCCCGGAACG TTCAGGGGGA GAGGAGGATG GGACGGATGGGG
1441 TATGACCCGG ACTCCTCCTG GCGGAGAGGAA GTGTTGTGAA UAGGTTCCCG GGTGAGGAGT
1501 ATGGACACTG CGGAGAGGAA GATGGGCTCT GTGCCCATGT GCAGCGTGGG GGCTGAGGGG
1561 TGGACCCAGTC TCGGCTGGCA GACACAGACT TAACTTATGT TCTATTTAAT GTGTAATTAT
1621 AGCCTGCCCC CCCCTTCCTT GTGTTTGGAG GTGCCCATGT GCAGCGTGGG GGCTGAGGGG
1681 TCCTGTGTGT ATCTTGGGGT TTCCCCACAT TAAAGCTGTT A TCCGG
```

FIG. 12

SEQ. ID NO. 3

cHB9-protein

```
  1  MEKSKNFRID  ALLAVIDPPKA  AAQSAPLALV  TGGSGGGSPP  SSSSSSSSSS  SSSSELPADC
 61  PRTDSPSPPR  LLPAHCALLP  KAAFLGGGGP  GGGHPQHHAL  GLHPAGPGGP  GLYGHPVYGY
121  PALGGQHPAL  SYSYSQVQGA  HPAHPSADPI  KLSAGTFQLD  QWLRASTAGM  LLPKMPDFGS
181  QAQSNLLGKC  RRPRTAFTSQ  QLLELEHQFK  LNKYLSRPKR  FEVATSLMLT  ETQVKIWFQN
241  RRMKWKRQKK  AKEQAAQEAE  NEKGGGGGED  KSGPRELLLP  GPEKGGGRRL  RELPDSEPED
301  EEEEEEEEEE  AEAGRCCPYH  SSDCSEADEE  DSQSGGRPGA  PPPPPAQPQ*
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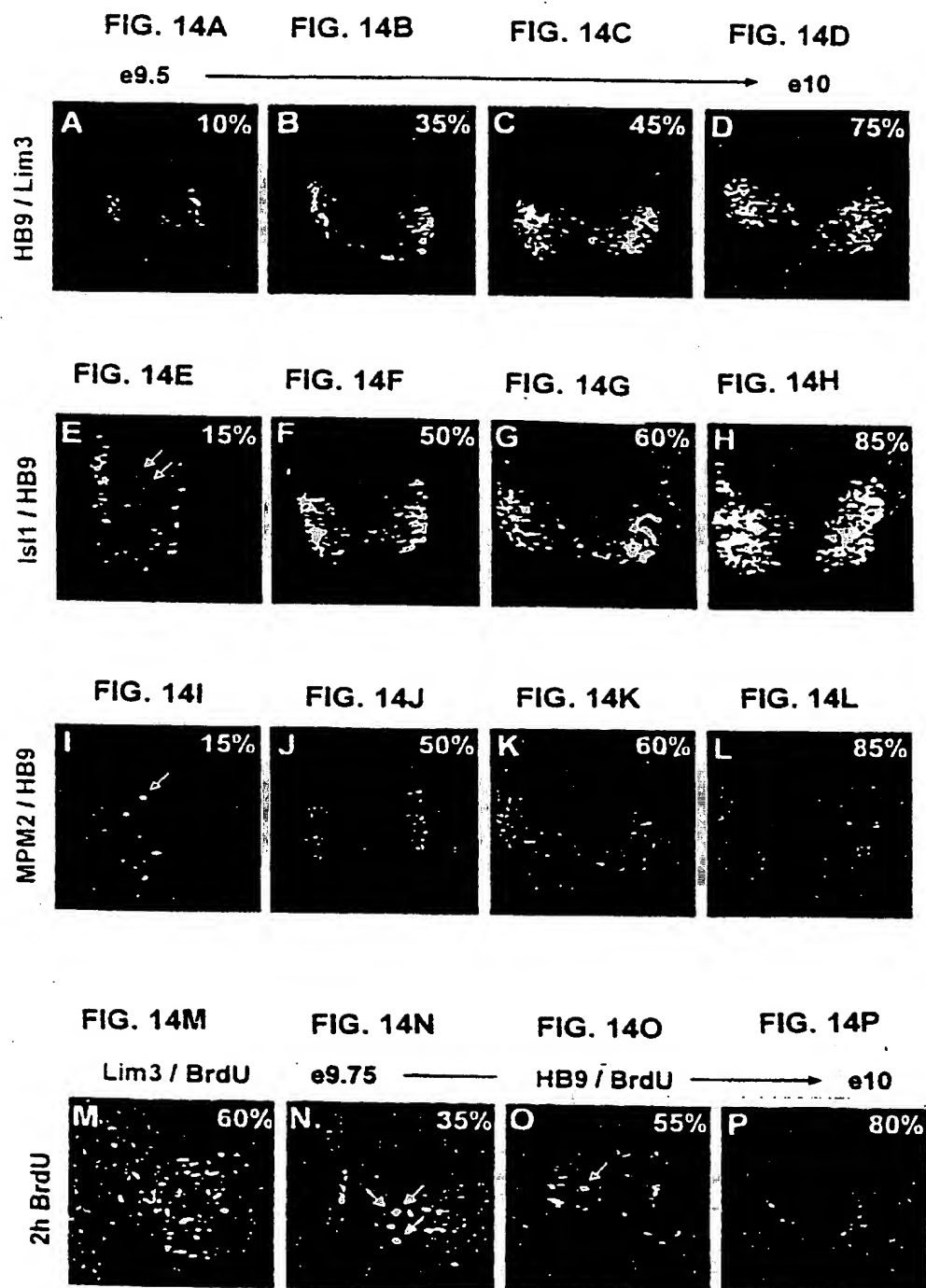
FIG. 13
SEQ. ID NO. 4

cHB9-DNA

```
   1  CCGGGCTGGC CTCTCGCCGC CTCCGCCGCT CCCATGGAAA AATCCAAAAA TTTCCGCATC
  61  GACGCGCTGC TGGCTGTCGA TCCCCCCAAG GCGGGGGCGC AGAGGCCTCC GCTGGCCCTG
 121  GTCACCGGCG GCTCCGGCGG CGGCAGCCCT CCGTCTTCGT CCGACAGCCC GTCTCCGCCT
 181  TCCTCCTCTT CTTCCGAGCT CCCGCGCCGAC TGCCCGGGCA CCGACAGCCC CTCTCCGCCT
 241  CGCCTGCTGC CCGCGCACTG CGCGCTGCTG CCCAAAGCCG CCTTCCTGGG CGGGGGCGGG
 301  CCCGGGGGCG GCCACCCGCA GCACCACGCC CTGGGCTGC ACCCCGGCGGG GCCGGGGCGGG
 361  CCGGGCCTCT ACGGGCACCC GGTGTACGGC TACCCGGCGT TGGGCGGGCA GCACCCGGCG
 421  CTCTCCTATT CCTATTCGCA AGTGCAGGGA GCGCACCCCG CGCATCCCTC CGCCGACCCC
 481  ATCAAGCTGA GCGCCGGCAC CTTTCAGCTG GACCAGTGGC TGCGGGCGAG CACGGCCGGC
 541  ATGATCCTGC CCAAATGCC TGACTTCGGC TCTCAGCCGC AGTTCCAACCT GCTGGGGAAG
 601  TGCCCGGCGGC CGCGCACCGC CTTCACCCAGC CAGCAGCTGC TGGAGCTGGA GCACCAGTTC
 661  AAACTCAACA AGTACCTCTC CCGGCCCAAG CGCTTCGAGG TGGCCCAGTC GCTGATGCTC
 721  ACCGAGACGC AGGTGAAGAT TTGGTTCCAG AACCGCCGCA TGAAATGGAA GCGGCCAGAAA
 781  AAGGCGAAGG AGCAGGCGGC GCAGGAGGCA GAGAACGAGA AAGGAGGAGG AGGAGGAGAG
 841  GACAAAAGCG GGCCGAGGGA ACTGCTGCTG CCCGGCCCGG AGAAAGGCGG CGGGGAGGCGG
 901  CTGAGGGAGC TGCCCGACAG CGAGCCCGAG GACGAGGAGG AGGAAGAAGA GGAGGAAGAG
 961  GAGGCCGAGG CCGGGCGGTG CTGCCCCTAC CACTCCTCCG ACTGCTCCGA GGCGGACGAG
1021  GAGGACTCGC AGTCCGGAGG ACGGCCCGGA GCCCCCCCGC CACCCCCCGC ACAGTCGCAG
1081  TGAGCCCACG GCCGCCCCGT CGGGGCGCC CCCGGGGCCC GAGCCTCCTG GCCCCGCTCT
1141  CCATCCCGCT CTCCCATCCC TCCCTGCTCG GAGGGGGACG CGGAAAGGGA TCTCCCGTCT
1201  GCCGAGGCGGG AGGGAGGATT CACACAGTGT TATTATTGAC TGAGAAGCGG CCACGACTTG
1261  AGCCCCCCTC CCCGCCCCGC CCTATCGGAA CCGTTTCCTT CTTACCCATAT ATCGGGAAAA
1321  GTGTTTATGT CATGAACGTT AAAACTGCTG CAGATCTCAA TACTGTCTTT ATTTGTCATA
1381  TCCTATTTAT AAAAAGGCA AATGAATTC CTCTACTTAT GCATGCTAAA TTATTACCCA
1441  GCCCCTTCCG CCTGAGGTGG GGGGGAGGAA TATAAATAAA GAGCGTTTTG TACTGTGAAA
1501  AAAAAAAAA AAAA
```

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FIG. 14A   FIG. 14B   FIG. 14C   FIG. 14D

e9.5 ──► e10

HB9 / Lim3

A   10%   B   35%   C   45%   D   75%

FIG. 14E   FIG. 14F   FIG. 14G   FIG. 14H

Isl1 / HB9

E   15%   F   50%   G   60%   H   85%

FIG. 14I   FIG. 14J   FIG. 14K   FIG. 14L

MPM2 / HB9

I   15%   J   50%   K   60%   L   85%

FIG. 14M   FIG. 14N   FIG. 14O   FIG. 14P

Lim3 / BrdU   e9.75 ─────   HB9 / BrdU ──────► e10

2h BrdU

M   60%   N   35%   O   55%   P   80%

FIG. 14Q

Position of MN, V2 neurons

Q

V2

MN

FIG. 14R

Mouse

R

Lim3
HB9
Isl1
Isl2

FIG. 14S

Chick

S

MNR2
Lim3
Isl1
HB9
Isl2

FIG. 15A

FIG. 15A

FIG. 15B

FIG. 15B

FIG. 15C   FIG. 15D   FIG. 15E   FIG. 15F:

FIG. 15C   FIG. 15D   FIG. 15E   FIG. 15F:

FIG. 15G   FIG. 15H   FIG. 15I

FIG. 15G   FIG. 15H   FIG. 15I

FIG. 16A -- Hb9+/- -- Hb9-/-

FIG. 16B nls-lacZ

FIG. 16C βGal / Isl

FIG. 16D Brn3.0 / βGal

FIG. 16E FIG. 16G Cervical FIG. 16I Lumbar

FIG. 16F FIG. 16H FIG. 16J

Hb9+/- Hb9-/-

FIG. 17A   FIG. 17B   FIG. 17C   FIG. 17D

FIG. 17E   FIG. 17F   FIG. 17G   FIG. 17H

FIG. 18A

Hb9+/-

FIG. 18B

Hb9-/-

FIG. 18C

FIG. 18D

FIG. 18E

FIG. 18F

FIG. 18G

Chx10 / HRP

FIG. 18I

Chx10 / Isl

FIG. 18K

Myc

FIG. 18M

NF160 / Myc

FIG. 18H

FIG. 18J

FIG. 18L

FIG. 18N
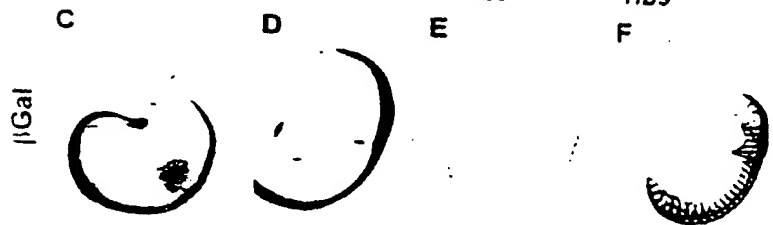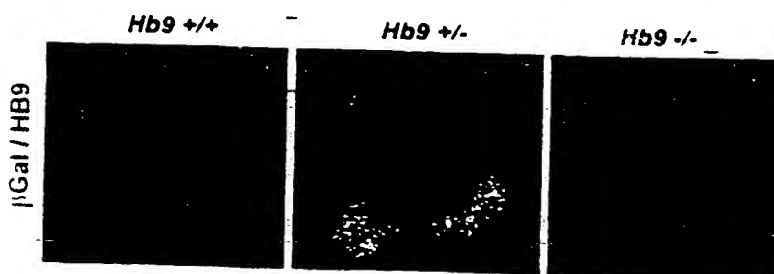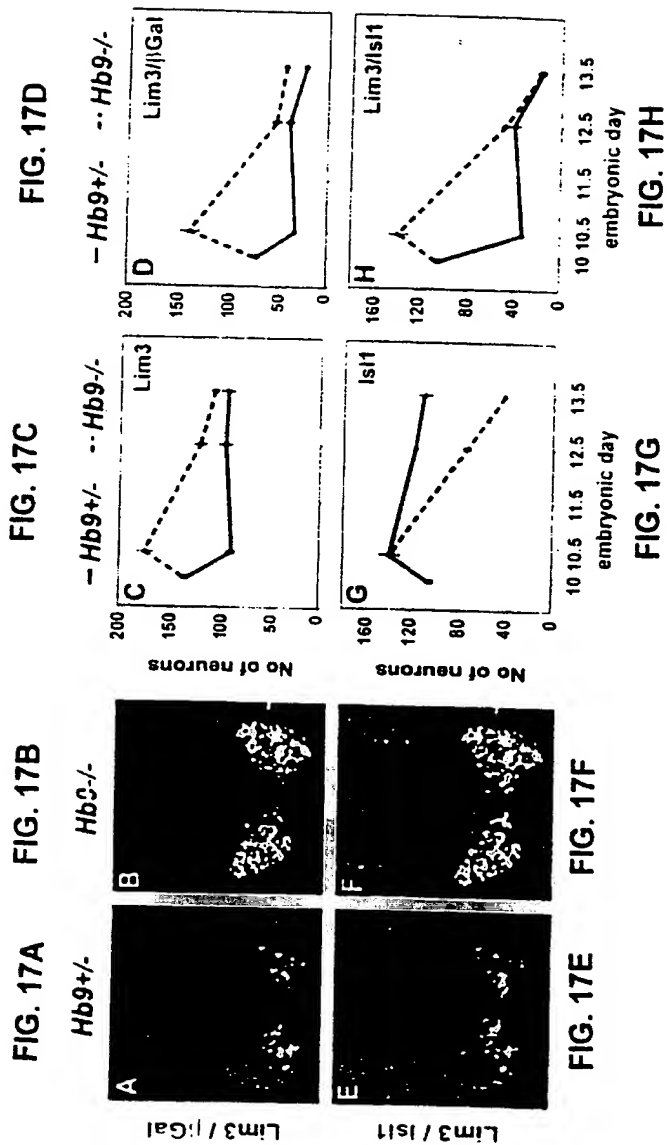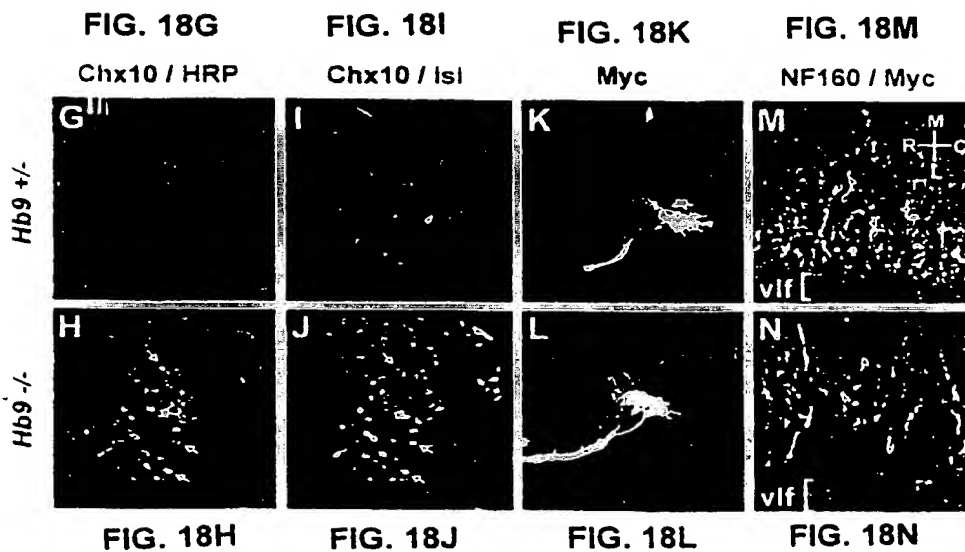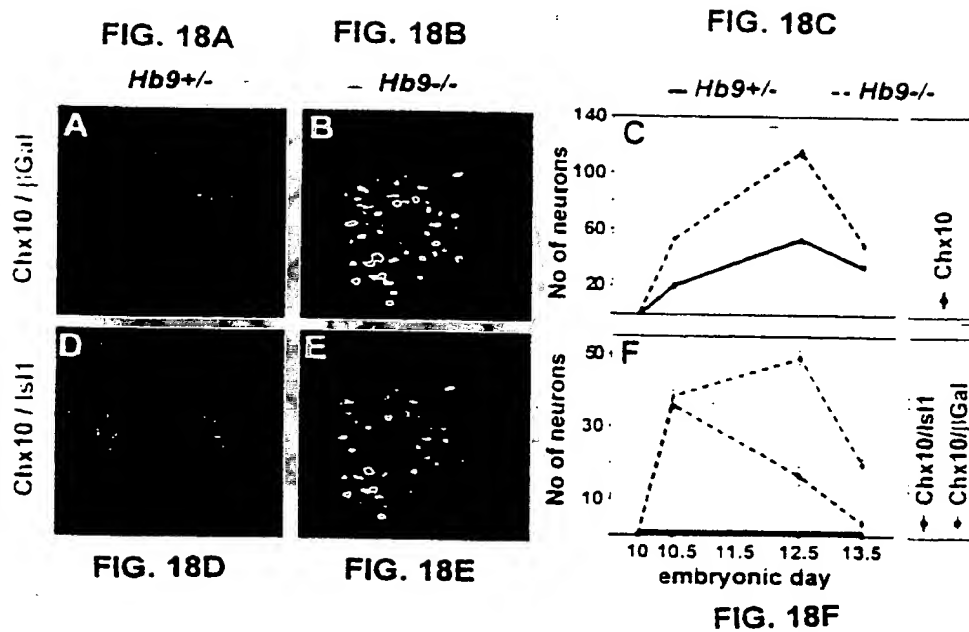
FIG. 19A

A   Progressive MN Specification
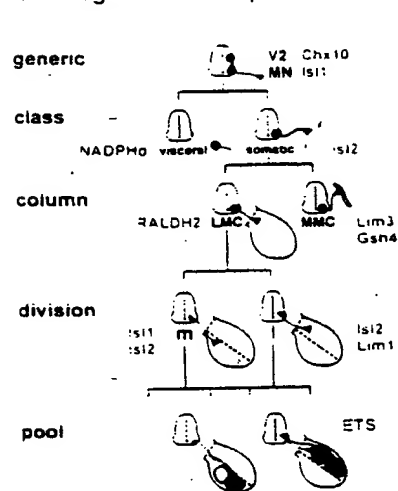
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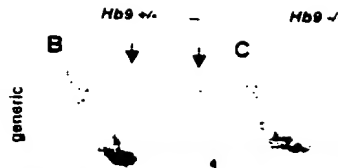
FIG. 19C
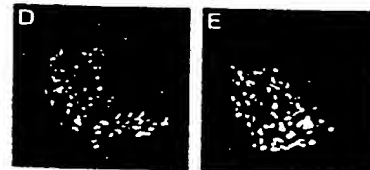
FIG. 19D

FIG. 19E

FIG. 19F

FIG. 19G

FIG. 19H

FIG. 19I

FIG. 19J

FIG. 19K

FIG. 19L

FIG. 19M

FIG. 19N

FIG. 19O

FIG. 20A   FIG. 20B   FIG. 20C   FIG. 20D

FIG. 20E   FIG. 20F   FIG. 20G   FIG. 20H

FIG. 20I   FIG. 20J   FIG. 20K   FIG. 20L

FIG. 21

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FIG. 22A   FIG. 22C   FIG. 22E

FIG. 22B   FIG. 22D   FIG. 22F

FIG. 22G   FIG. 22H   FIG. 22I

FIG. 23I
FIG. 23J
FIG. 23G
FIG. 23H
FIG. 23E
FIG. 23F
FIG. 23C
FIG. 23D
FIG. 23A
FIG. 23B

FIG. 24A FIG. 24B FIG. 24C FIG. 24D FIG. 24E FIG. 24F FIG. 24G FIG. 24H FIG. 24I FIG. 24J

FIG. 25A
FIG. 25B
FIG. 25C
FIG. 25D
FIG. 25E
FIG. 25F
FIG. 25G
FIG. 25H
FIG. 25I
FIG. 25J
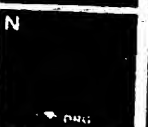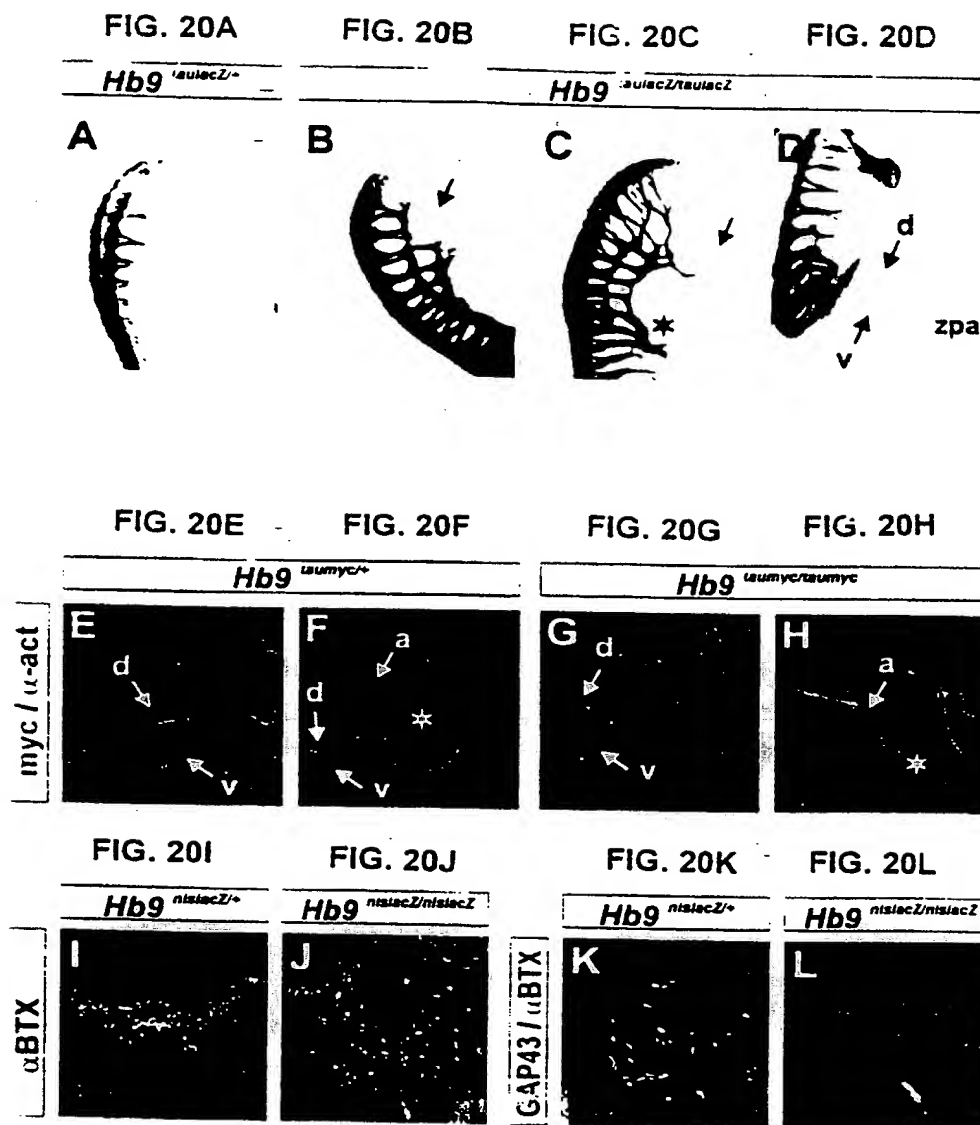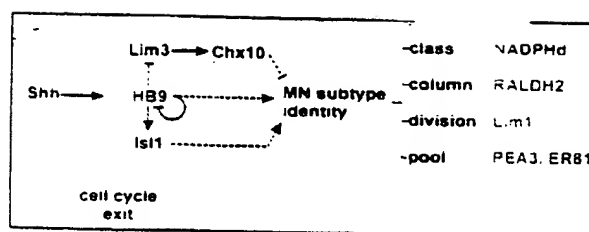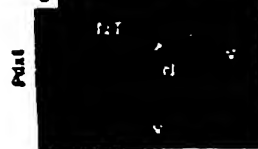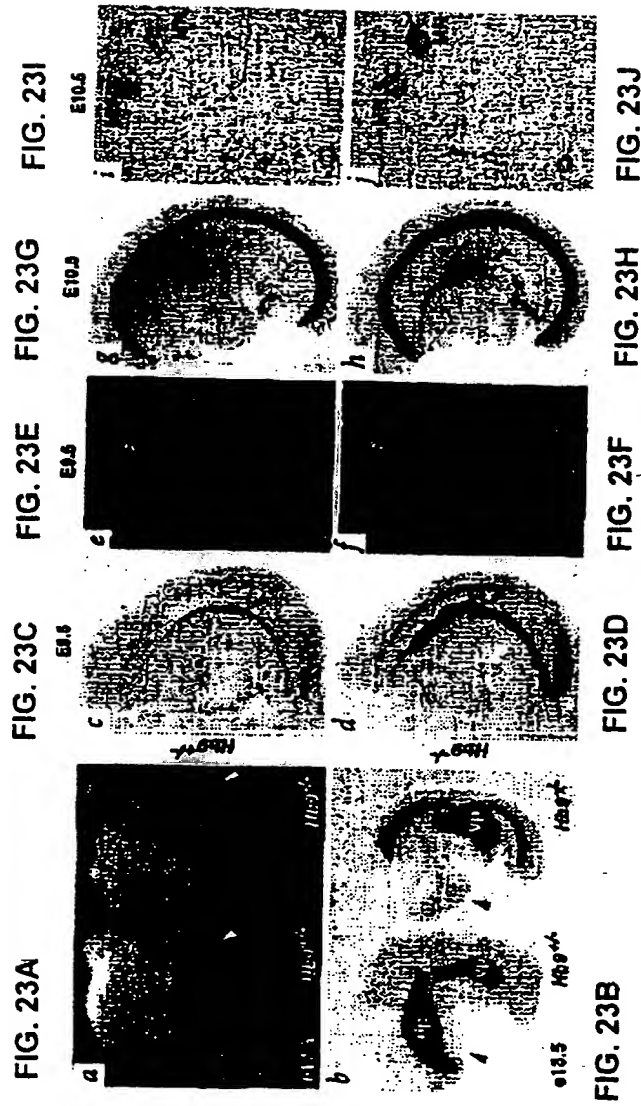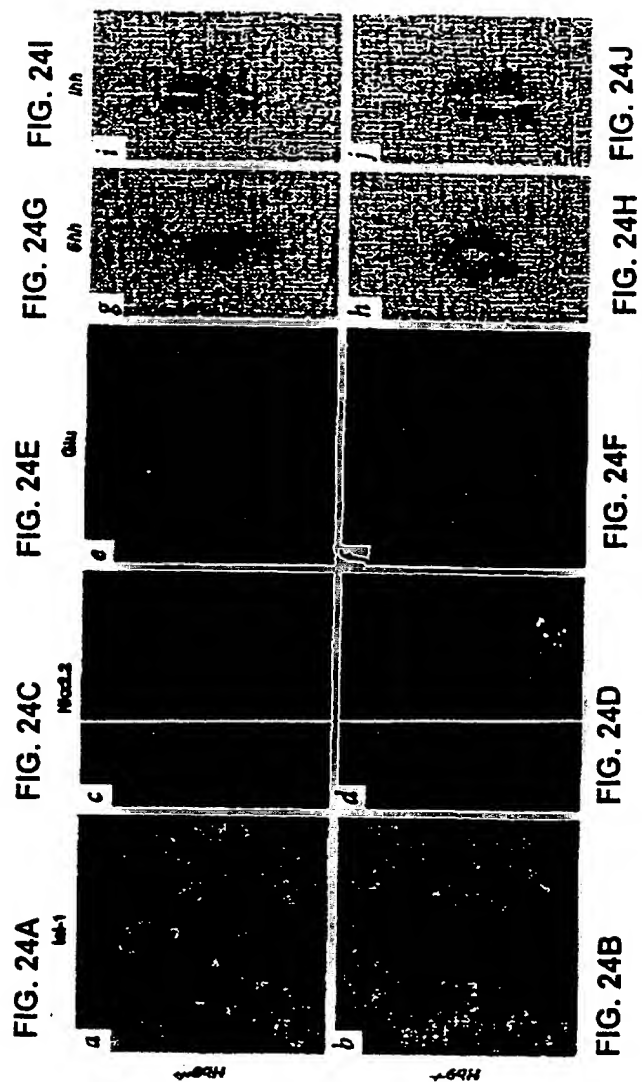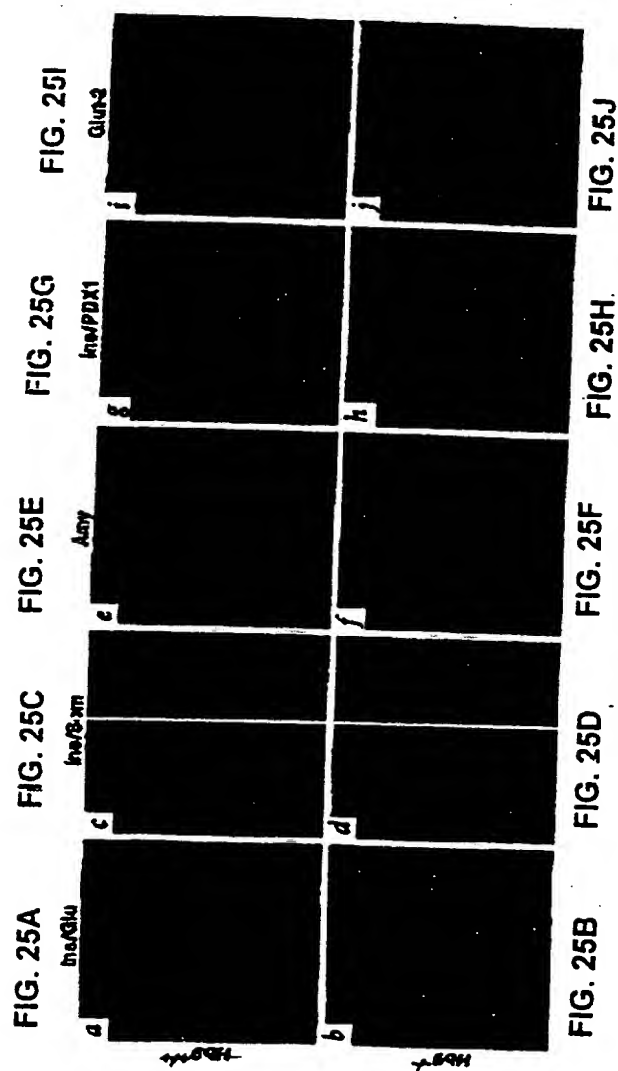
INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/22517

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   435/69.1, 252.3, 320.1, 325, 419; 536/23.5, 24.31, 24.5; 530/350, 387.1; 800/8

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN: MEDLINE CAPLUS BIOSIS EMBASE BIOTECHDS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X ---- A, X	TANABE et al. Specification of motor neuron identity by the MNR2 homeodomain protein. Cell. October 1998, Vol. 95, pages 67-80, entire document.	1-7,9,14-17 27-33, 34-40, 44, 48 ----------- 8, 10-13, 41-43, 45-47, 49-60
X -- Y	Database Tumor Gene Index. Accession No. AI560820. NCI-CGAP. National Cancer Institute, Cancer Genome Anatomy Project (CGAP), Tumor Gene Index. Entire document, especially bases 50-129. June 1998.	18-23 --------- 24-26

X	Further documents are listed in the continuation of Box C.		See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier document published on or after the international filing date
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O"	document referring to an oral disclosure, use, exhibition or other means
"P"	document published prior to the international filing date but later than the priority date claimed

"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 NOVEMBER 1999	25 JAN 2000

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RICHARD SCHNIZER Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y -- A	HARRISON et al. A novel human homeobox gene distantly related to *proboscipedia* is expressed in lymphoid and pancreatic tissues. Journal of Biological Chemistry. August 1994, Vol. 269, No. 31, pages 19968-19975, entire document, especially materials and methods.	61-68, 71, 74-76, 78-89 ------------ 101, 102, 108 ------------ 69, 98-100, 103, 104-107, 109-124
X -- Y -- A	DEGUCHI et al. Nucleotide sequence of a novel diverged human homeobox gene encodes a DNA binding protein. Nucleic Acids Research. 1991, Vol. 19, No. 13, page 3742, entire document.	61-64, 68, 71,72,74, 75,78-83 ------------ 84-89,101,102,104, 108 ------------ 76, 98-1 00,103,105-107, 109-124
X -- Y -- A	SAHA et al. Dorsal-ventral patterning during neural induction in *Xenopus*: Assessment of spinal cord regionalization with *xHB9*, a marker for the motor neuron region. Developmental Biology. July 1997, Vol. 187, pages 209-223, entire document.	61-68, 74, 75, 78-83 ------------ 84-86, 101, 102, 104, 108 ------------ 69, 76, 98-100, 103, 105-107, 109-124
A, P	ARBER et al. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron. August 1999. Vol. 23, No. 4, pages 659-674, entire document.	61-89, 98-124
A, P	THALER et al. Active suppression of interneuron programs with developing motor neurons revealed by analysis of homeodomain factor HB9. Neuron. August 1999, Vol 23, No. 4, pages 675-687, entire document.	61-89, 98-124

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 13, 70, 73, 77, and 90-97
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

   Please See Extra Sheet.

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                        ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

C12N 1/21, 15/00, 15/11, 15/63, 15/85, 15/86; C07H 21/02, 21/04; C07K 14/00, 16/00; A01K 67/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 252.3, 320.1, 325, 419; 536/23.5, 24.31, 24.5; 530/350, 387.1; 800/8

BOX I. OBSERVATIONS WHERE CLAIMS WERE FOUND UNSEARCHABLE
2. Where no meaningful search could be carried out, specifically:

Claim 13 is drawn to the nucleotide sequence of SEQ ID No. 1. SEQ ID No.1 is not a nucleotide sequence, it is a chick MNR2 amino acid sequence. Claims 70, 73, 77, and 90-97 recite amino acid or nucleotide sequences of an HB9 protein or gene as encoded by SEQ ID No.1. SEQ ID No.1 is not an HB9 amino acid or nucleotide sequence, it is a chick MNR2 amino acid sequence.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claim(s)1-12, 14-26, and 44, and drawn to MNR2 nucleic acids, vectors and host cells comprising them, and a first method of using the nucleic acids to produce an MNR2 protein.
Group II, claim(s) 27-33, 48-55 and 60, drawn to a purified MNR2 protein, and methods of using it.
Group III, claim(s) 34-37, drawn to anti-MNR2 antibodies.
Group IV, claim(s) 41 and 42, drawn to a nonhuman transgenic animal comprising an MNR2 transgene and a method of using it.
Group V, claim(s) 56 and 57, drawn to a second method of using MNR2 nucleic acids, to diagnose neurological disorders.
Group VI, claim(s) 58 and 59, drawn to unidentified compounds of unknown structure which are functional analogs of MNR2.
Group VII, claim(s) 61-69, 71, 72, 74-76, 78-86 and 104, drawn to HB9 nucleic acids, vectors and host cells comprising them, and a first method of using them to produce HB9 protein.
Group VIII, claim(s) 87-89, 108, and 120-124, drawn to a purified HB9 protein and methods of using it.
Group IX, claim(s) 101-102, drawn to a transgenic animal comprising an HB9 transgene and a method of using it.
Group X, claim(s) 116, and 117, drawn to a second method of using HB9 nucleotides, to diagnose neurological disorders.
Group XI, claim(s) 118 and 119, drawn to unidentified compounds of unknown structure which are functional analogs of HB9.

The inventions listed as Groups I-XI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I is drawn to a group of products, specifically all MNR2 nucleic acids. Groups II-IV, VI-IX and XI are drawn to independent products, and group V is drawn to a second method of using the invention of group I. 37 C.F.R. 1.475(b) does not provide for multiple independent products, and only provides for a first method of using the product. Group X is drawn to a method of using a product which is independent of the product of group I.

Therefore the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single general inventive concept.

Form PCT/ISA/210 (extra sheet)(July 1992)*